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TITLE THE PROTEOLYTIC PROCESSING OF ORGANELLAR PROTEINS

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THE PROTEOLYTIC PROCESSING OF ORGANELLAR PROTEINS

Diane C. Bassham

A Thesis Submitted for the Degree of
Doctor of Philosophy
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Coventry
U.K.

SUMMARY

Biogenesis of the chloroplast involves the activities of both the nuclear and chloroplast genomes. Nuclear-encoded chloroplast proteins are synthesised on free ribosomes in the cytosol and imported into the chloroplast post-translationally. Protein uptake is directed by an N-terminal presequence which is removed after import by specific processing peptidases. Thylakoid lumen proteins must cross three membranes to reach their site of function and have a composite presequence to enable their correct localisation, consisting of an envelope transit domain (ETD) and a thylakoid transfer domain (TTD). The ETD is removed after translocation across the envelope by a stromal processing peptidase (SPP) to produce an intermediate-sized protein and the TTD then allows transport into the thylakoid lumen where it is removed by a thylakoidal processing peptidase (TPP). SPP is a soluble, stromal-located metallopeptidase which also removes the presequences from stromal proteins. Despite its apparently high specificity, there is very little sequence similarity around SPP cleavage sites in stromal and thylakoidal proteins, and little is known about the structural features which SPP recognises.

SPP was partially purified from pea chloroplasts and the SPP cleavage site within the presequences of three thylakoid lumen proteins was determined by radiosequencing of the cleavage products with the two-fold aim of identifying the residues around the cleavage site which SPP may recognise and of delineating the ETD and TTD regions of the presequences, allowing their comparison with signal sequences. This information was then used to create four mutant thylakoid lumen precursor proteins by site-directed mutagenesis with altered SPP processing sites. The four mutant proteins all showed a reduced rate and efficiency of processing in an organelle-free time course assay, although all were imported into chloroplasts, correctly localised and processed to the mature size in an *in vitro* assay.

Although SPP has usually been considered to be highly specific for imported chloroplast precursor proteins, it has recently been suggested that it may also process mitochondrial precursor proteins, which are cleaved *in vivo* and *in vitro* to the mature size by the mitochondrial processing peptidase (MPP). These two peptidases were therefore compared in terms of substrate specificity and mechanism by assaying the activity of the two enzymes against chloroplast and mitochondrial precursor proteins. MPP did not process any of the chloroplast precursor proteins available, suggesting that this enzyme is indeed highly specific for mitochondrial precursor proteins. A partially-purified SPP preparation cleaved the mitochondrial precursor proteins tested to a smaller size; however, the site of cleavage in at least some of these proteins was different to the authentic MPP cleavage site, with the stromal preparation cleaving N-terminal to MPP. This stromal processing activity was not due to mitochondrial contamination and evidence from inhibitor sensitivities and column chromatography suggests that SPP cleaves both chloroplast and mitochondrial precursor proteins. The SPP processing site within the presequences of two mitochondrial proteins was determined by radiosequencing and compared with the chloroplast cleavage sites, and this data should enable further analysis to determine the features which constitute a site which can be recognised by SPP.

An SPP activity was also partially-purified from *Chlamydomonas reinhardtii* and shown to be located in the stroma. This activity is able to process chloroplast precursor proteins from *C. reinhardtii* and pea to an intermediate or mature size, emphasising the similarity of the specificity of SPP from these two species.

CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

ACP	acyl carrier protein
ADP	adenosine diphosphate
ala	alanine
amp	ampicillin
arg	arginine
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
$b_2\Delta19$	truncated cytochrome b_2-DHFR fusion protein
BiP	binding protein
bis-acrylamide	N'N'-methylene-bisacrylamide
bp	base pairs
BSA	bovine serum albumin
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CD	circular dichroism
cDNA	complementary DNA
CFoII	chloroplast Fo ATP synthase subunit II
CIP	calf intestinal alkaline phosphatase
cm	centimetre
CoA	coenzyme A
cox	cytochrome oxidase
cpm	counts per minute
C. reinhardtii	*Chlamydomonas reinhardtii*
C-terminal	carboxyl-terminal
cys	cysteine
cyt	cytochrome
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate

dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
DCMU	dichlorophenyldimethylurea
DEAE-	diethylaminoethyl-
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTNB	5,5'-dithio-bis-(2-nitrobenzoic) acid
DTT	1,4-dithiothreitol
E-64	*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
ECL	enhanced chemi-luminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(aminoethyl ether)N,N'-tetraacetic acid
EPSP	5'-enolpyruvylshikimate-3-phosphate
E.R.	endoplasmic reticulum
ETD	envelope transit domain
F_1ATPase	mitochondrial F_1 ATPase
$F_1\beta$	F_1ATPase β subunit
FAD	flavine adenine dinucleotide
FPLC	fast protein liquid chromatography
Fd	ferredoxin
Fe/S	Rieske iron-sulphur protein

FNR	ferredoxin-NADPH reductase
g	grams
g	unit of gravitational field
GIP	general insertion protein
glu	glutamic acid
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
his	histidine
hr	hour
hsp	heat shock protein
i-	intermediate
IAA	iodoacetic acid
ile	isoleucine
IM	inner membrane
IMP I	inner membrane protease I
IMS	intermembrane space
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kDa	kilo-Dalton
l	litre
LDC	lysine decarboxylase
LEP	leader peptidase
leu	leucine
LHCP	light-harvesting chlorophyll *a/b* binding protein
LSP	prolipoprotein signal peptidase
LSU	Rubisco large subunit
lys	lysine
M	molar
μCi	microCuries

µE	microEinsteins
µg	micrograms
µl	microlitres
µmol	micromoles
mA	milliamperes
MBP	maltose binding protein
MDa	megaDaltons
met	methionine
mg	milligrams
MIP	mitochondrial intermediate peptidase
ml	millilitres
mM	millimolar
MOM	mitochondrial outer membrane protein
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
M.STET	sucrose-Tris-Cl-EDTA-Triton X-100
mt	mating type
MW	molecular weight
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NEM	N-ethylmaleimide
N. crassa	*Neurospora crassa*
ng	nanograms
nm	nanometres
nM	nanomolar
nmol	nanomoles
NMR	nuclear magnetic resonance

nptII	neomycin phosphotransferase II
N-terminal	amino-terminal
OAA	oxaloacetic acid
OD	optical density
O. danica	*Ochromonas danica*
OEC	oxygen-evolving complex
OM	outer membrane
o-phen	1,10-phenanthroline
OTC	ornithine transcarbamylase
PBF	presequence binding factor
PBS	phosphate-buffered saline
PC	plastocyanin
PEG-6000	polyethylene glycol-6000
PEP	processing enhancing protein
PGA	3-phosphoglycerate
pmf	proton motive force
pmol	picomoles
PMSF	phenylmethylsulphonylfluoride
PQ	plastoquinone
Pre-	precursor
PSI	photosystem I
PSII	photosystem II
P680	reaction centre of PSII
P700	reaction centre of PSI
rATP	adenosin ribonucleotide triphosphate
rCTP	cytidine ribonucleotide triphosphate
rGTP	guanosine ribonucleotide triphosphate
rUTP	uridine ribonucleotide triphosphate
RER	rough E.R.

RNA	ribonucleic acid
RNase A	ribonuclease A
RNasin	ribonuclease inhibitor
rpm	revolutions per minute
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	second
ser	serine
SIM	sucrose isolation medium
SP	signal peptidase
spermidine	N-[3-aminopropyl]-1,4-butanediamine
SPP	stromal processing peptidase
SRM	sorbitol resuspension medium
SRP	signal recognition particle
SSU	Rubisco small subunit
TAP	Tris-acetate-phosphate
TBE	Tris-borate-EDTA
TBq	TeraBequerel
TCA	trichloroacetic acid
TE	Tris-Cl-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	Tris-Cl-EDTA-sucrose-lysozyme
thr	threonine
TPP	thylakoidal processing peptidase
Tris	tris(hydroxymethyl)methylamine
Triton X-100	*iso*-octylphenoxypolyethoxyethanol
TTD	thylakoid transfer domain
Tween 20	polyoxyethylenesorbitan monolaurate

tyr	tyrosine
u.v.	ultraviolet
val	valine
v/v	volume/volume
w/v	weight/volume
x-gal	5-bromo-4-chloro-3-indolyl β-D-galactoside
^{35}[S]met	^{35}sulphur labelled methionine
^{3}[H]leu	tritium labelled leucine
^{3}[H]lys	tritium labelled lysine
^{3}[H]phe	tritium labelled phenylalanine
16K	16 kDa OEC protein
23K	23 kDa OEC protein
33K	33 kDa OEC protein

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DECLARATION

The work presented in this thesis is original work conducted by myself under the supervision of Dr C. Robinson. This research was funded by an SERC studentship and Schering Agrochemicals. All sources of information have been acknowledged by means of reference. None of the work has been used in any previous application for a degree.

I am grateful to the following people for their help: Dr J. Meadows (Warwick) provided a cDNA clone encoding wheat pre-33K. H. James (Warwick) provided a cDNA clone encoding wheat pre-23K. Dr P. Weisbeek provided a clone encoding *Silene pratensis* pre-PC. Prof J.-D. Rochaix provided cDNA clones encoding *Chlamydomonas reinhardtii* precursor proteins. Antisera to $F_1\beta$ and cDNA clones encoding pre-cyclophilin, pre-$F_1\beta$, pre-$b_2\Delta19$, pre-MPP and pre-Fe/S were provided by Prof W. Neupert (Munich). MPP and PEP were provided by Dr. M. Arretz (Munich). Maize mitochondria were provided by A. Liddell (Oxford). Microsequencing of cleaved proteins was carried out in collaboration with B. Dunbar (Aberdeen). A. Creighton (Warwick) provided part of figure 45.

CHAPTER 1 - LITERATURE REVIEW

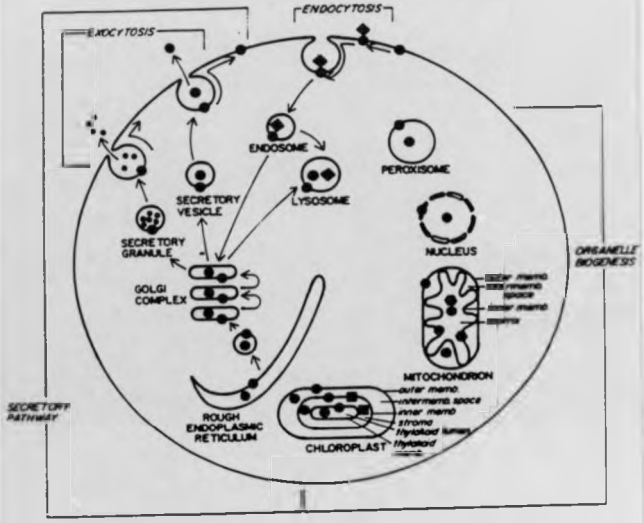
1.1 Introduction

The eukaryotic cell contains many membrane-bound organelles which allow the compartmentalisation of the biochemical reactions of the cell. Each compartment has specific functions to perform, and so must contain a specific set of enzymes and other proteins to carry out these functions; for example, the mitochondrion carries out oxidative phosphorylation, the chloroplast photosynthesis, the lysosome protein degradation and the peroxisome cellular respiration and fatty acid oxidation. The cell therefore is presented with the problem of directing proteins, most of which are synthesised in the cytosol, to the required compartment for their function (figure 1).

Proteins destined for secretion from the cell are synthesised on polysomes bound to the cytosolic face of the endoplasmic reticulum (ER) membrane (Blobel and Dobberstein, 1975). The newly-synthesised proteins are translocated co-translationally across the ER membrane into the lumen, from which they are transported through the secretory system by a process of vesicle budding and fusion, with no additional membrane translocation step (Rothman and Orci, 1992). Nuclear-encoded proteins destined for other organelles such as mitochondria, chloroplasts and peroxisomes are synthesised on free polysomes in the cytosol and must be directed post-translationally to their site of function (Wickner and Lodish, 1985). Mechanisms are therefore required for the targeting of proteins to the correct compartment both co- and post-translationally, and for sorting to specific subcompartments within each organelle. These often involve an amino-terminal presequence which is removed after targeting of the protein by a specific peptidase. The aim of this work was to study the proteolytic processing of nuclear-encoded chloroplast proteins after import into the chloroplast.

Figure 1 *Schematic diagram of protein targeting within the eukaryotic cell*

The sites to which proteins are targeted within a eukaryotic cell are shown. The secretory pathway transports nuclear-encoded proteins (●) through the endoplasmic reticulum and Golgi apparatus to lysosomes or to the cell surface. Chloroplasts and mitochondria, in addition to containing many nuclear-encoded proteins which are imported from the cytosol, contain a small genome which encodes a subset of chloroplast (■) or mitochondrial (●) proteins. Proteins synthesised by other cells (◆) can be imported into the cell by endocytosis, and directed to lysosomes for degradation or to other intracellular compartments. (Taken from "Protein Targeting", by A. P. Pugsley, 1989)


ENDOCYTOSIS
EXOCYTOSIS
ENDOSOME
PEROXISOME
SECRETORY VESICLE
LYSOSOME
SECRETORY GRANULE
NUCLEUS
ORGANELLE BIOGENESIS
GOLGI COMPLEX
MITOCHONDRION
outer membr.
intermembr. space
inner membr.
matrix
SECRETORY PATHWAY
ROUGH ENDOPLASMIC RETICULUM
CHLOROPLAST
outer membr.
intermembr. space
inner membr.
stroma
thylakoid lumen
thylakoid

1.2 Structure and Functions of the Chloroplast

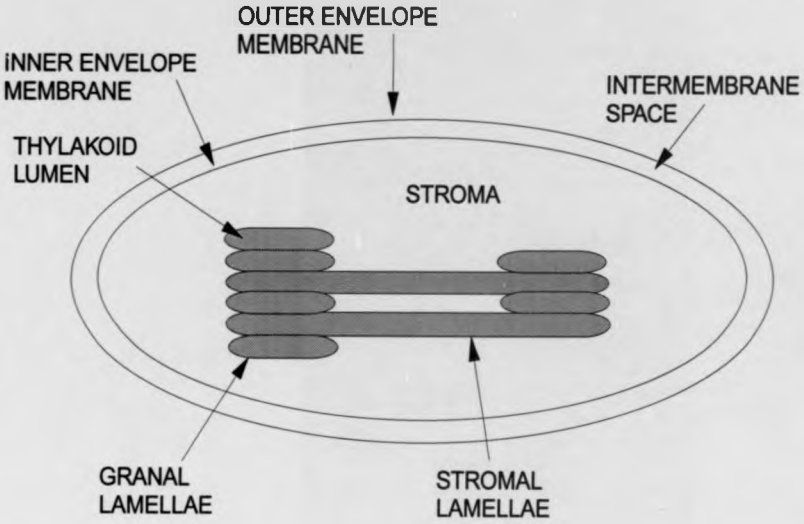
The chloroplast is a member of the plastid family of organelles and contains the characteristic green pigment chlorophyll and a small genome of between 82 and 96 MD_d (Lawlor, 1987), along with the machinery required for protein synthesis within the chloroplast. Chloroplasts cannot be formed *de novo* but divide during cell division with a concomitant replication of the chloroplast DNA (Hoober, 1984). The higher plant chloroplast is a particularly complex structure as shown in figure 2, consisting of three distinct membranes, the outer and inner envelope membranes and the thylakoid membrane, which enclose three soluble phases, the intermembrane space, the stroma and the thylakoid lumen.

(a) Envelope

The chloroplast envelope consists of the outer and inner envelope membranes, which enclose the intermembrane space, and its main functions are in the uptake of molecules from the cytosol and in lipid biosynthesis. The outer membrane is permeable to molecules of molecular weight (MW) less than about 10,000 and is a major site of lipid synthesis (Douce and Joyard, 1990). The inner membrane is a selective barrier and so contains a series of specific translocators. Each of the chloroplast membranes has a characteristic lipid and protein content reflecting their various functions. All three membranes contain a high proportion of glycolipids and a low proportion of phospholipids, with phosphatidylcholine found exclusively in the outer membrane. Several proteins are also found only in one of the envelope membranes, for example the outer membrane contains acylCoA synthetase whereas the inner membrane contains galactosyl transferase and phosphatidic acid phosphatase. Little is known of the functions of the intermembrane space.

Figure 2 *Structure of the higher plant chloroplast*

A schematic diagram showing the six phases of the chloroplast. The chloroplast envelope consists of the outer and inner envelope membranes enclosing the intermembrane space. The envelope surrounds another soluble phase, the stroma, in which is the thylakoid network comprising the soluble thylakoid lumen enclosed by the thylakoid membrane.


OUTER ENVELOPE MEMBRANE
INNER ENVELOPE MEMBRANE
INTERMEMBRANE SPACE
THYLAKOID LUMEN
STROMA
GRANAL LAMELLAE
STROMAL LAMELLAE

(b) Stroma

The stroma is the site of several of the biosynthetic reactions of the chloroplast, containing the dark reaction enzymes for carbon dioxide reduction to produce carbohydrates, amino acids and fatty acids. The major stromal enzyme is ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) which consists of eight chloroplast-encoded large subunits which contain the catalytic site and eight nuclear-encoded small subunits whose function is unclear, but may have a regulatory role. Rubisco catalyses the first step of the dark reactions, the conversion of carbon dioxide and ribulose bisphosphate to 3-phosphoglycerate (PGA). PGA is reduced to phosphoglyceraldehyde in a reaction which utilises the NADPH and ATP formed during the photosynthetic light reactions, and the phosphoglyceraldehyde can be transported out of the chloroplast via the phosphate translocator for sucrose synthesis in the cytosol, or converted into starch within the stroma. Other important reactions include the synthesis of fatty acids using ATP, NADPH and carbohydrates and the reduction of nitrite to ammonia using the reducing power of light-activated electrons to provide the nitrogen required for amino acid and nucleotide biosynthesis (Hall and Rao, 1987). The stroma also contains the chloroplast DNA and the protein synthesising machinery.

(c) Thylakoid Network

The thylakoid network is arranged as a series of stacked discs or granal lamellae which are connected by single stromal lamellae, with the thylakoid lumen thought to be a single continuous phase. The thylakoid membrane contains the four major protein complexes involved in photosynthesis; photosystems I and II (PS I and PS II), cytochrome *b*$_6$*f* and the chloroplast ATP synthase (figure 3).

Figure 3 *The major protein complexes of the thylakoid membrane*

A schematic representation of the protein complexes identified in higher plant thylakoid membranes. Photosystem II, cytochrome *b6/f* complex, photosystem I and the ATP synthase complex are shown. The molecular weights of the proteins in barley thylakoids are indicated. (Taken from "Photosynthesis", by D. O. Hall and K. K. Rao, 1987).

~60Å
T H Y L K K O D
57kD
58kD
55kD
35 kD
21kD
IX 19 kD
15 kD
II
DCCD proton channel
(-6) 8kD
I 19kD
LHCI 680
20kD
25kD
LHCI 730
21kD
22kD
24kD
110kD Chl$_a$-P1
FNR 44kD
A_2
A_1
110kD Chl$_a$-P1
P700
15kD
21kD
8kD
11kD Fd
PC 11kD
33kD cyt f
cyclic electron flow
19.5kD Rieske fe-S
17kD
23.5kD cyt b_6
23.5kD cyt b_6
Chl$_{a/b}$-P2 (LHCII)
POH$_2$
32kD Chl$_a$-P1
32kD D1
O_A O_B
34kD D2
DCMU
Chl$_a$-P2
oxep
53kD
P680
47kD Chl$_a$-P3
33kD
Chl$_{a/b}$-P2 28,25,23kD
10kD cyt b_{559}
22kD
10kD
24kD
23kD
16kD

(d) The Light Reactions of Photosynthesis

The role of photosynthesis is the light-driven synthesis of carbohydrates from CO_2 and water. The light reactions of photosynthesis result in the transfer of electrons from water to $NADP^+$, with the evolution of oxygen, and the transfer of hydrogen ions across the thylakoid membrane into the thylakoid lumen to form a proton motive force (pmf; figure 4). This pmf then drives protons back across the membrane through the chloroplast ATP synthase complex to produce ATP.

The photosynthetic electron donor is water, which is oxidised to O_2 by the oxygen-evolving complex (OEC) of PS II. The final electron acceptor is $NADP^+$, which is reduced to NADPH by PS I, the overall reaction being

$$2\ H_2O + 2\ NADP^+ \Rightarrow O_2 + 2\ NADPH + 2\ H^+$$

PS I contains the electron donor P700 which may consist of a dimer of chlorophyll *a*, which is bound to one or both of two central chloroplast-encoded polypeptides, A1 and A2. When P700 is excited by photons of light, initially absorbed by the light harvesting complexes, its midpoint potential becomes more negative and this causes an electron to be ejected. This electron reduces the first known acceptor, A_0, which may be a type of chlorophyll, and then A_1, which is thought to be vitamin K_1 or a similar compound. The next electron acceptors are iron-sulphur centres, of which PS I has three, F_X, F_A and F_B. F_X is thought to reside on the large A1 and A2 polypeptides, while F_A and F_B have been assigned to a 9 kDa polypeptide. Electrons are transfered from PS I to ferredoxin (Fd), which reduces $NADP^+$ via the Fd-$NADP^+$ reductase and FAD and so completes the electron transport. Cyclic electron transport may also occur if levels of $NADP^+$ are low, with electrons being returned from Fd to the plastoquinone pool via cytochrome *b6/f*. This pathway generates ATP but not NADPH (Andréasson and Vänngård, 1988). PS II (figure 5) contains the core proteins D1 and D2 which bind the electron donor P680 (thought to consist of chlorophyll *a*), phaeophytin *a*, and two quinones, Q_A and Q_B. The reaction centre also contains a 43 kDa and 47 kDa protein, which are thought to have multiple roles including the binding of antenna chlorophyll, maintenance of PS

Figure 4 *The Z-scheme showing the pathway of electron flow during photosynthesis*

The pathway of electron transfer in chloroplasts in relation to redox potential is shown. The two photosystems cooperate, via a series of electron carriers, in the reduction of $NADP^+$ using electrons extracted from water. Abbreviations used are:

Q	bound quinone
PQ	plastoquinone pool
f	cytochrome *f*
PC	plastocyanin
Fd	ferredoxin

(Adapted from "Bioenergetics : an introduction to the chemiosmotic theory", by D. G. Nicholls, 1982)

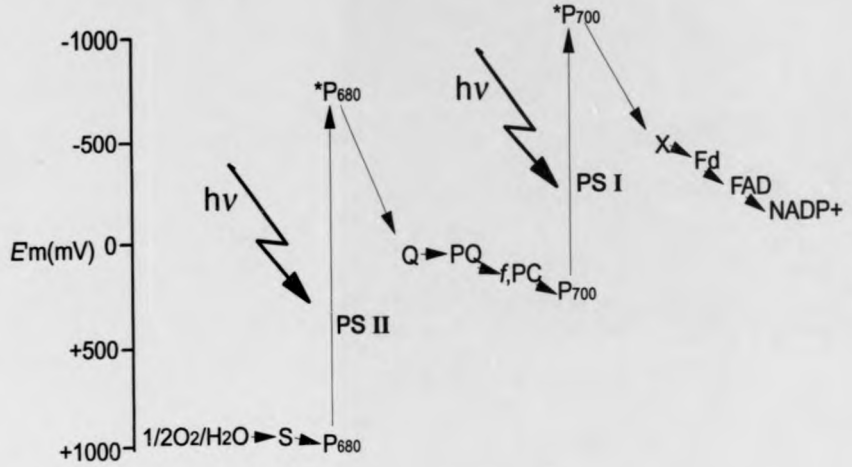
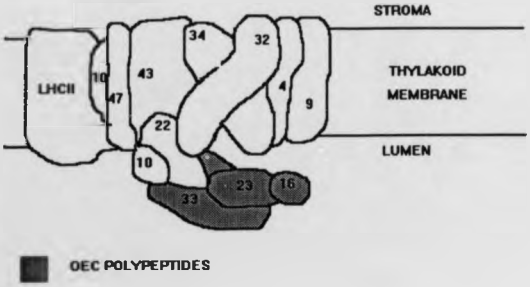

-1000
-500
E'm(mV) 0
+500
+1000
hν
PS II
*P680
1/2O2/H2O
S
P680
Q
PQ
f,PC
P700
hν
PS I
*P700
X
Fd
FAD
NADP+

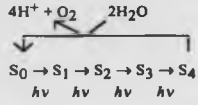
Figure 5 *Model of the photosystem II complex of higher plants*

The photosystem II complex consists of a core complex, light harvesting complex and an oxygen evolving complex (shaded). The oxygen evolving complex consists of three peripheral membrane proteins of 16, 23 and 33 kDa. Numbers on the proteins indicate their molecular weights.


STROMA
34
32
LHCII
10
43
47
THYLAKOID
MEMBRANE
4
9
22
LUMEN
10
23
16
33
OEC POLYPEPTIDES

II structure and regulation of quinone and manganese binding. Cytochrome b_{559} co-purifies with D1 and D2 but its function is unknown. P680 is the primary electron donor of the PS II reaction centre which donates an electron to phaeophytin *a*. The electron is transferred to the bound quinones Q_A and Q_B and a bulk pool of plastoquinone links PS I to plastocyanin (PC) via the cytochrome b_6/f complex. This complex may be involved in what is known as the 'Q-cycle' in which the plastoquinone donates an electron pair to the complex, one of the electrons being transferred to PC via a Reiske iron-sulphur protein and cytochrome *f* and the other transferred to cytochrome b_{563}. It has been suggested that this electron is then transferred to the outside of the thylakoid membrane where two electrons and two protons from the stroma reduce a bound plastoquinone molecule which dissociates and joins the pool of reduced plastoquinone. The cycling of electrons therefore doubles the number of protons transferred across the membrane per electron transported through the chain. PC can then re-reduce PS I to allow further rounds of electron transport (Lawlor, 1987).

PS II also contains three extrinsic proteins which make up the OEC, which is situated on the lumenal face of the thylakoid membrane, the 33 kDa (33K), 23 kDa (23K) and 16 kDa (16K) proteins. 33K probably binds to the 47 kDa intrinsic protein and possibly to the D1/D2/cytochrome b_{559} complex, whilst 23K binds to a component of the thylakoid membrane and also to 33K, and 16K probably binds to 23K. 33K is probably involved in stabilising manganese binding but the functions of 23K and 16K are unknown. Oxygen evolution is a linear 4 electron oxidation reaction described by the model:



where S_0 - S_4 are the different oxidation states of S. PS II contains four atoms of manganese per reaction centre and spectroscopic measurements suggest that a Mn (III) is oxidised to Mn (IV) in each of the first three S-state transitions. A tyrosine residue within the D1 polypeptide is thought to be an intermediate electron acceptor which transfers electrons from water to P680. The splitting of water generates protons which accumulate in the thylakoid lumen to produce a pH gradient across the thylakoid membrane. The pmf generated is used to drive ATP synthesis by the CF_0F_1 ATP synthase complex.

The OEC also contains Cl^- and Ca^{2+} inorganic co-factors. PS II activity is inhibited by Cl^- depletion, although the role of Cl^- in oxygen evolution is unknown. Ca^{2+} may bind to 23K close to the manganese cluster, affecting the structure of the cluster. The role of the OEC proteins may be to increase the binding of Cl^- and Ca^{2+} ions and also to protect the manganese cluster from external reducing agents (Ghanotakis and Yocum, 1990).

The OEC proteins are all nuclear-encoded and are transported post-translationally into the chloroplast, where they are proteolytically processed. The 33K protein was used during this project to study this processing event.

1.3 Transport of Proteins Into and Within Chloroplasts

The biogenesis of the chloroplast requires the activities of both the nuclear and chloroplast genetic systems. Some chloroplast proteins are encoded by the chloroplast genome and are synthesised in the stroma, whereas the majority are nuclear-encoded, synthesised in the cytosol and transported post-translationally into the organelle. Nuclear-encoded proteins can be found in all of the compartments of the chloroplast. Chloroplast-encoded proteins are found in the stroma and thylakoids, and a recent report described the detection of the first chloroplast-encoded envelope protein (Sasaki *et al.*, 1993). Several chloroplast enzyme complexes contain both nuclear- and chloroplast-encoded subunits, suggesting that the synthesis and/or degradation of these subunits must be tightly coupled (Schmidt and Mishkind, 1983).

1.3.1 Transport of Proteins Across the Chloroplast Envelope Membranes

Most early studies of chloroplast protein import involved the import of nuclear-encoded stromal proteins, in particular the small subunit of rubisco (SSU), due to its high abundance. Dobberstein *et al.* (1977) reported that this protein is synthesised as a larger precursor (pre-SSU), and the specific post-translational import of pre-SSU into isolated intact chloroplasts, with cleavage of the precursor to the mature size, was demonstrated by Chua and Schmidt (1978) and Highfield and Ellis (1978). Translocation across the envelope can be monitored by inaccessibility to added protease (Highfield and Ellis, 1978) or incorporation into active complexes (Chua and Schmidt, 1979; Smith and Ellis, 1979).

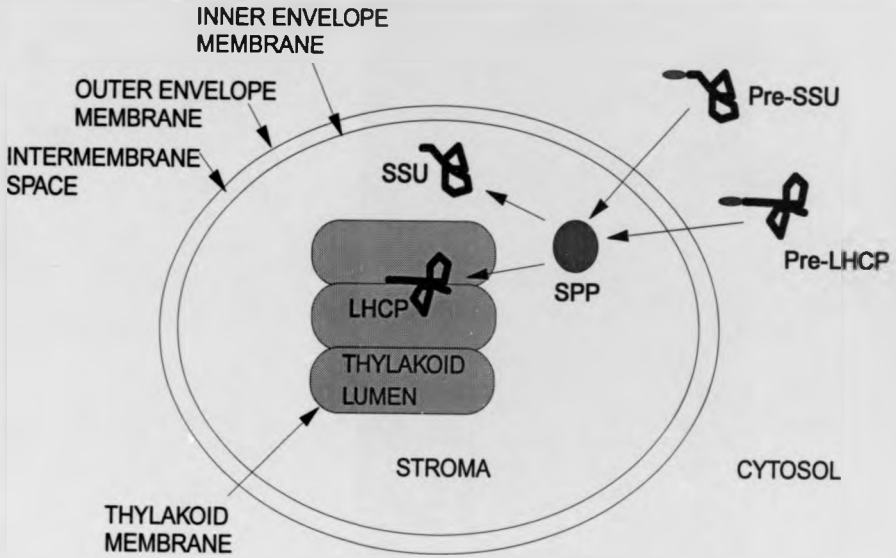
Some proteins are present in both the cytosol and chloroplast of the same cell. In most cases, proteins in different locations are encoded by different genes, with the chloroplast isoform being synthesised with a presequence directing it into the chloroplast (*eg* glutamine synthetase; Lightfoot *et al.*, 1988). Pyruvate orthophosphate dikinase, however, has stromal and cytosolic forms that are thought to be encoded by a single gene, with alternative mRNA processing pathways generating the different forms (Aoyagi and Bassham, 1984).

(a) Binding of Precursor Proteins to the Chloroplast Envelope

Precursor proteins are thought to be imported after specific binding to receptors in the chloroplast envelope membranes (figure 6). Cline *et al.* (1985) protease-treated intact chloroplasts before incubation with precursor proteins and this led to a reduction in import and binding of both pre-SSU and pre-LHCP (light harvesting chlorophyll *a/b* binding protein), suggesting that a protein of the chloroplast envelope which was susceptible to proteases is involved in import. Friedman and Keegstra (1988) showed that the binding of pre-SSU to the envelope is saturable, with 1500 - 3500 binding sites per chloroplast and saturation reached at about 80 nM precursor. Competition studies using a synthetic presequence, however, showed that a very high level of peptide (160 μM) was needed to completely block

Figure 6 *Import pathway of pre-SSU and pre-LHCP into chloroplasts*

Proteins are synthesised in the cytosol as precursors with N-terminal presequences and transported post-translationally into the chloroplast stroma. The presequences are removed by the stromal processing peptidase (SPP) to generate the mature sized protein, followed by localization of pre-LHCP to the thylakoid membrane.


INNER ENVELOPE MEMBRANE
OUTER ENVELOPE MEMBRANE
INTERMEMBRANE SPACE
Pre-SSU
SSU
Pre-LHCP
LHCP
SPP
THYLAKOID LUMEN
STROMA
CYTOSOL
THYLAKOID MEMBRANE

protein import (Buvinger *et al.*, 1989). This could reflect a reduced binding affinity of the presequence compared with the complete protein. Pre-SSU from *Chlamydomonas reinhardtii* bound to intact chloroplasts and isolated envelope membranes from *C. reinhardtii* with equal affinity, and this binding was shown to be specific for chloroplast membranes and dependent on the presence of a presequence (Su *et al.*, 1992). Peptides corresponding to the C-terminal thirty amino acid residues of the pre-Fd and pre-SSU presequences were each able to block the binding and import of both full precursors into chloroplasts, suggesting a common import receptor (Schnell *et al.*, 1991), and *E. coli*-produced purified pre-LHCP was found to inhibit the import of radiolabelled pre-LHCP and pre-SSU, suggesting that pre-LHCP and pre-SSU also share some components of the receptor or import apparatus (Oblong and Lamppa, 1992). A role for lipids in the binding and import of pre-SSU has also been implied as phospholipase C treatment of chloroplasts completely abolished import, whilst binding to the envelope was increased by this treatment (Kerber and Soll, 1992). The bound precursor was still protease sensitive, and it was suggested that the transfer of precursor from the receptor to the import apparatus was blocked in this case. Fragments of the presequence of rubisco SSU were found to be surface active and able to insert into lipid monolayers, with a preference for chloroplast envelope lipids (van't Hof *et al.*, 1991). Pre-ferredoxin also efficiently inserts into an outer envelope membrane extract, the interaction requiring the presence of the presequence and a loose lipid headgroup packing (van't Hof *et al.*, 1993). As the interaction of pre-ferredoxin with lipids was found to be specific for chloroplast outer envelope membrane lipid extract and that of the mitochondrial cytochrome *c* oxidase subunit IV presequence to be specific for a mitochondrial outer membrane lipid extract, a role for lipids in the targeting specificity of presequences was suggested. It has also been suggested that the lipid bilayer could undergo a structural rearrangement during protein translocation to allow proteins to pass directly through the membrane (Keegstra, 1989), with this rearrangement being induced by the membrane active presequence.

Dobberstein *et al.* (1977) proposed that chloroplast proteins were translocated across the envelope at contact sites between the inner and outer membranes. Immunofluorescence microscopy has provided evidence for the localisation of putative receptor proteins and precursor protein translocation intermediates at sites where the two envelope membranes are closely apposed (Pain *et al.*, 1988; Schnell *et al.*, 1990; Schnell *et al.*, 1991; Schnell and Blobel, 1993) and analogies with mitochondrial protein import (Wienhues *et al.*, 1991) suggest that this may be the case.

Several putative chloroplast import receptors have been identified. A 51 kDa envelope membrane protein is phosphorylated during protein translocation (Hinz and Flügge, 1988) and has been suggested to be part of the receptor complex. Cornwell and Keegstra (1987) used a cross-linking approach to identify a putative receptor of approximately 66 kDa involved in the import of pre-SSU. Cross-linking using a synthetic presequence led to the identification of two proteins of 52 kDa and 30 kDa (Kaderbhai *et al.*, 1988). The 52 kDa protein is probably the large subunit of rubisco (LSU), and the 30 kDa protein was proposed to be an import receptor. A 30 kDa protein was also identified as a putative import receptor by Pain *et al.* (1988) using a different approach. Antibodies were raised against the carboxy-terminal 30 amino acids of the pre-SSU presequence and used to produce anti-idiotypic antibodies. These anti-idiotypic antibodies inhibited the import of pre-SSU into pea chloroplasts and bound to a 30 kDa protein at contact sites between the outer and inner chloroplast envelope membranes. The sequence of this protein was found to have a high homology to the chloroplast phosphate translocator from spinach (Flügge *et al.*, 1989) and later to be identical to the sequence of the pea phosphate translocator (Willey *et al.*, 1991). Flügge *et al.* (1989) demonstrated the ATP-dependent insertion of the spinach phosphate translocator into the chloroplast envelope and showed that it was processed and protease protected, which would not be expected for a receptor at protein import sites. Schnell *et al.* (1990) raised antibodies against the 30 kDa putative import receptor and these were shown to inhibit chloroplast protein import.

The binding of this protein to hydroxylapatite was used as further evidence that it was an import receptor and not the phosphate translocator, which had previously been shown not to bind to hydroxylapatite (Flügge and Heldt, 1981). Flügge *et al.* (1991) replied with the suggestion that the differences in hydroxylapatite binding were due to different experimental conditions and that the phosphate translocator may share some common epitopes with an import receptor, explaining the inhibition of protein import by antibodies against the 30 kDa protein. A putative mitochondrial receptor protein has also been found to be homologous to two putative phosphate translocators from animal mitochondria (Murakami *et al.*, 1990). They also provided evidence that the 30 kDa protein is an inner envelope membrane protein, rather than an outer membrane protein as may be expected for a receptor. The 30 kDa protein could therefore be an import receptor, phosphate translocator or both, with further work needed to resolve this issue.

(b) Translocation of Precursor Proteins Across the Chloroplast Envelope

The energy requirements for import across the envelope have been studied in some detail. Import was shown to be energy-dependent by Grossman *et al.* (1980) as translocation could be stimulated by light or the addition of ATP. Pretreatment of chloroplasts with the uncoupler nigericin prevented import of precursors into the chloroplast, but still allowed binding to the chloroplast surface to occur (Cline *et al.*, 1985). Bound precursor could be chased into the chloroplast by the addition of ATP, showing that ATP is essential for import. Pain and Blobel (1987) demonstrated that ATP is required in the stroma for protein translocation across the envelope and the hydrolysis of ATP, probably in the intermembrane space, was later shown to be required for high affinity binding (Olsen *et al.*, 1989). A low concentration of ATP is now thought to be required in the intermembrane space for the binding of precursors to the chloroplast surface and a higher concentration in the stroma for protein translocation across the envelope, with no requirement for a pmf across the envelope membranes (Theg *et al.*, 1989).

Cytosolic factors may be essential for the import of some precursor proteins, as *E. coli* expressed purified pre-LHCP could only be imported into chloroplasts after dialysis in the presence of leaf extract (Waegemann *et al.*, 1990). This effect could be partially compensated for by hsp 70, suggesting that the import of this protein requires the presence of hsp 70 and at least one other cytosolic factor. This does not appear to be the case for all imported proteins, as ferredoxin was shown to be import competent in the absence of added cytosol (Pilon *et al.*, 1990), with import dependent only on added ATP (Pilon *et al.*, 1992).

Soll and Waegemann (1992) isolated a functionally active protein import complex after solubilisation of outer envelope membranes and separation of membrane complexes by sucrose density gradient centrifugation. This membrane complex interacted with precursor proteins in a reaction dependent on the presence of a presequence, ATP and protease-sensitive components, and produced translocation intermediates with similar properties to those produced during import across intact membranes. The identification of more components of these import complexes, and studies involving depletion and reconstitution of components, should shed more light on the mechanisms involved in the translocation of precursor proteins across the envelope membranes.

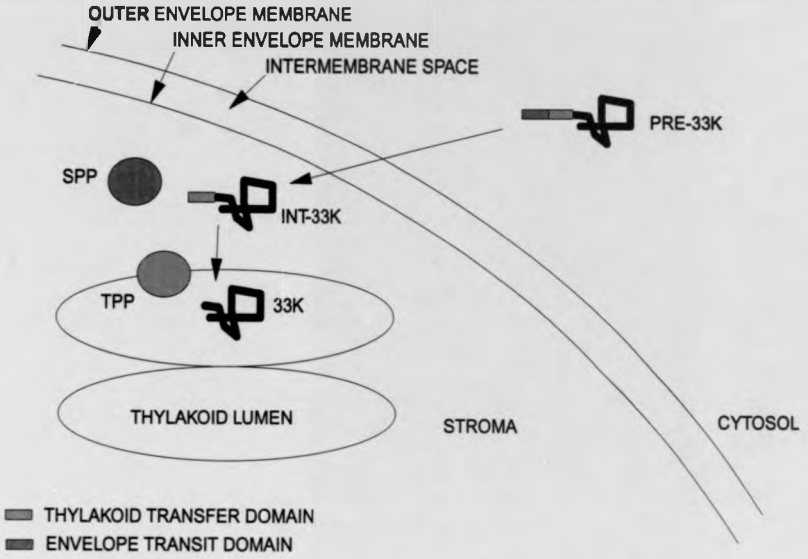
After or during translocation of precursors across the envelope membranes the presequence (or the N-terminal domain of a thylakoid lumen protein presequence) is removed by a stromal processing peptidase (SPP) to produce the mature (or intermediate) sized protein (Robinson and Ellis, 1984a). This enzyme is described in more detail in section 1.3.8(a). Proteins may then be assembled into their active complexes, in many cases with the involvement of the chloroplast chaperonin 60 to prevent mis-folding and aid assembly (Lubben *et al.*, 1989).

1.3.2 Translocation of Proteins Across the Thylakoid Membrane

After translocation of precursor proteins across the envelope membranes, these proteins must be sorted to their correct intra-organellar compartment. For thylakoid lumenal proteins, this involves a further membrane translocation step as the proteins must cross the thylakoid membrane. Initial work on the translocation of proteins across the thylakoid membrane concentrated on plastocyanin (PC), a small, soluble electron carrier, as this was the first lumenal protein for which a cDNA clone was available (Smeekens *et al.*, 1985). Plastocyanin was found to be located in the thylakoid lumen after import into isolated intact pea chloroplasts but a fusion protein consisting of the plastocyanin presequence fused to the mature ferredoxin protein, lacking the final plastocyanin processing site, was found as an intermediate-sized protein retained in the stroma (Smeekens *et al.*, 1986). Based on these observations, a two-step import model was proposed for the transport of thylakoid lumenal proteins (figure 7). It was suggested that thylakoid lumenal proteins are synthesised with a presequence consisting of two domains; an N-terminal domain (envelope transit domain, ETD) which is responsible for targeting the precursor across the envelope membranes, and a C-terminal domain (thylakoid transfer domain, TTD) which directs transport across the thylakoid membrane. The ETD would thus be equivalent to the presequence of stromal proteins. After translocation across the envelope membranes into the stroma, the ETD is removed by SPP to produce an intermediate-sized protein, which is further targeted to the thylakoid lumen, where the TTD is removed. This second processing step is catalysed by a thylakoid-located enzyme, the thylakoidal processing peptidase (TPP; Hageman *et al.*, 1986) and results in the production of the mature protein. Additional evidence for the two-step import model was provided by observations that the precursors of the 33, 23 and 16 kDa proteins of the OEC, which are lumenal proteins loosely bound to the thylakoid membrane, are also imported into isolated chloroplasts by a mechanism involving sequential cleavage by SPP in the stroma and TPP in the thylakoid lumen (James *et al.*, 1989; Ko and Cashmore, 1989). Hageman *et al.* (1990) used the PC ETD and TTD individually to show that they are

Figure 7 *Two-step model for the import of thylakoid lumen proteins into the chloroplast*

Thylakoid lumen proteins such as pre-33K are synthesised with a bipartite presequence. The envelope transit domain targets the precursor into the stroma where it is removed by the stromal processing peptidase. Translocation into the thylakoid lumen is directed by the thylakoid transfer domain, which is removed by the thylakoidal processing peptidase to produce the mature sized protein.


OUTER ENVELOPE MEMBRANE
INNER ENVELOPE MEMBRANE
INTERMEMBRANE SPACE
PRE-33K
SPP
INT-33K
TPP
33K
THYLAKOID LUMEN
STROMA
CYTOSOL
THYLAKOID TRANSFER DOMAIN
ENVELOPE TRANSIT DOMAIN

able to function independently of each other. The ETD was shown to be equivalent to a stromal targeting signal and could be replaced by a Fd presequence with no effect on the function of the presequence.

The development of a system for importing proteins into isolated thylakoids allowed the study of translocation across the thylakoid membrane in isolation from the envelope translocation system (Kirwin *et al.*, 1989). Import was found to be stimulated by the addition of ATP and a stromal extract and the imported protein (pre-33K) was processed to the mature size and resistant to added proteases. More efficient import of both pre-33K and pre-23K was found to require light in the place of ATP (Mould *et al.*, 1991), with pre-33K, but not pre-23K, requiring stromal extract. The modification of the 23K precursor protein by iodoacetate completely prevented cleavage by SPP but still allowed import of the full precursor into isolated thylakoids. These results were taken as indicating that the isolated thylakoids can import both the precursor and intermediate forms of 23K but only the intermediate form of 33K. The ability of the TTD of pre-33K to act as an internal targeting signal, however, was demonstrated by the export of pre-33K and processing to the mature size when expressed in *E. coli* (Meadows and Robinson, 1991). This also demonstrates the similarities between the components involved in translocation across the thylakoid and bacterial cytoplasmic membranes and between the presequences of thylakoid and bacterial proteins. The 16K (Klösgen *et al.*, 1992) and PC (Bauerle and Keegstra, 1991) precursors were later found to be efficiently imported into thylakoids as full-length precursor proteins, with no requirement for prior cleavage by SPP. Pulse-labelling experiments with *Chlamydomonas reinhardtii*, however, demonstrated the occurrence *in vivo* of intermediate forms of thylakoid lumen proteins, thus suggesting that cleavage by SPP of at least some thylakoid lumen proteins does occur *in vivo* before transport into thylakoids (Howe and Merchant, 1993).

The basis for the stimulation by light or ATP of protein uptake into thylakoids was suggested to be due to the production of a membrane potential across the thylakoid membrane. This was studied by using electron transport inhibitors and

ionophores to collapse the membrane potential (Mould and Robinson, 1991). Complete dissipation of the pmf using the electron transport inhibitors dichlorophenyldimethylurea (DCMU) and methyl viologen showed that transport across the thylakoid membrane required a pmf. The selective dissipation of the electrical potential ($\Delta\Psi$) by valinomycin/KCl or the proton gradient (ΔpH) by nigericin/KCl demonstrated that the ΔpH component of the pmf is the component required for translocation of 23K, 33K (Mould and Robinson, 1991) and 16K (Klosgen *et al.*, 1992), and depletion of the $\Delta\Psi$ had little effect on protein import. The import of 23K and 16K into thylakoids was found to be maximal in the complete absence of ATP (Cline *et al.*, 1992), indicating that the ΔpH can provide all of the energy required to transport these two proteins across the thylakoid membrane.

1.3.3 Targeting of Proteins to the Thylakoid Membrane

Most of the research involving protein targeting to the thylakoid membrane has concentrated on the major light-harvesting chlorophyll *a/b* protein of photosystem II (LHCP), an integral thylakoid membrane protein involved in light energy capture and in the stacking of the thylakoid grana (Mullet, 1983). Pre-LHCP is nuclear-encoded and is cleaved *in vivo* after transport into the chloroplast, often giving multiple products (Clark and Lamppa, 1992): for example, pre-LHCP from wheat is cleaved on import into chloroplasts to produce two polypeptides, of MW 25,000 and 26,000. In an organelle-free processing assay, only the 25 kDa product is seen (Abad *et al.*, 1989). The specific integration of pre-LHCP into the thylakoid membrane was demonstrated *in vitro* in chloroplast lysates, with correct integration being demonstrated by localisation to the thylakoids, resistance to NaOH extraction and partial protease protection. Integration was dependent on the presence of ATP and stromal extract, but independent of light (Cline, 1986). The precursor itself, rather than the mature protein, was the predominant form integrated into the membrane, suggesting that insertion and processing are independent events. This was also observed by Chitnis *et al.* (1986) who showed that pre-LHCP could be imported into

barley etioplasts, followed by assembly into the light-harvesting complex of photosystem II as either the precursor or processed form. The ratio of the two forms was dependent on the developmental stage of the etioplasts, with increased greening corresponding to an increase in the amount of processed form. Chlorophyll was therefore proposed to be required for the assembly of pre-LHCP into the light-harvesting complex *in vivo* (Kohorn *et al.*, 1986). However, work using a chlorophyll *b*-less mutant (Chitnis *et al.*, 1988) suggested that this was not the case and the dependence on the developmental stage of the plastids to be due to the developmentally-regulated appearance of a stromal factor required for insertion (Chitnis *et al.*, 1987). This stromal factor was shown to be a protein which acted in maintaining the solubility and insertion competence of the pre-LHCP (Payan and Cline, 1991), and was identified as a chloroplast member of the 70 kDa family of heat shock proteins (hsp70; Yalovsky *et al.*, 1992).

Upon deletion of the presequence of pre-LHCP (Viitanen *et al.*, 1988), the precursor could no longer be imported by isolated chloroplasts, showing that the presequence was essential for translocation across the envelope membrane, but integration into isolated thylakoid membranes was unaffected. Hand *et al.* (1989) exchanged the presequences of pre-LHCP and pre-SSU with no effect on localisation of the proteins after import into isolated chloroplasts. The presequence is thus a stroma-targeting signal, with the information for targeting to the thylakoid membrane residing in the mature protein. Mutagenesis of pre-LHCP showed that a putative α-helical membrane-spanning domain close to the C-terminus was essential for stable insertion (Kohorn and Tobin, 1989). The fusion of this domain to the C-terminus of pre-SSU caused insertion of the fusion protein into the thylakoid membrane, although full insertion of this domain required other regions of LHCP. Pre-LHCP is currently thought to be imported into chloroplasts by a presequence-mediated pathway similar to that of stromal proteins: *E. coli*-synthesised pre-LHCP will inhibit import of both pre-LHCP and pre-SSU into isolated chloroplasts (Oblong and Lamppa, 1992a), suggesting that early stages in the import pathway such as receptor binding are

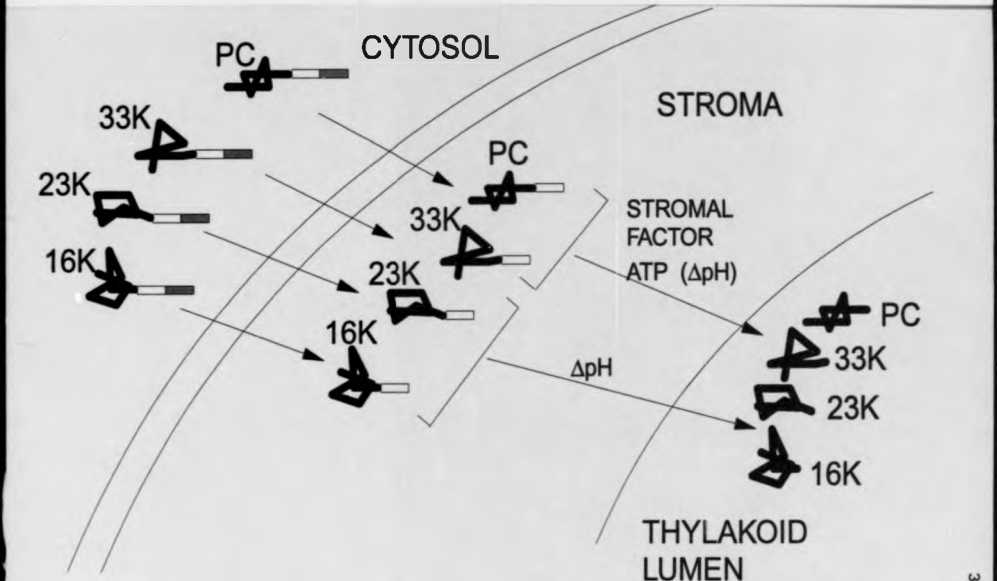
common to both proteins. Pre-LHCP is then inserted into stromal lamellae in a presequence-independent process and migrates to the granal lamellae where it is assembled into the light-harvesting complex of photosystem II (Yalovsky *et al.*, 1990), with processing to the mature size occuring at some point during this pathway (figure 6).

Another precursor whose import and assembly into the thylakoid membrane is similar to that of pre-LHCP is that of the 20 kDa apoprotein of the chlorophyll *a/b* antenna complex CP24 of photosystem II, a remote relative of LHCP. The protein precursor contains a presequence which directs translocation into the stroma and is not involved in the subsequent steps of integration into the thylakoid membrane, binding of chlorophyll, assembly into the CP24 complex and migration to the granal lamellae of the membrane (Cai *et al.*, 1993). All the steps of targeting and assembly of this protein can still occur if the authentic presequence is replaced by a stromal protein presequence.

Unlike pre-LHCP, the precursor to subunit 2 of the chloroplast F_0 ATPase (pre-$CF_0$2), also an integral thylakoid membrane protein, is synthesised with a presequence which appears very similar in structure to a thylakoid lumen protein presequence *i.e.* containing both an ETD and a TTD. This precursor is not a substrate for the stromal processing peptidase which removes the ETD from thylakoid lumen proteins and if transport to the thylakoid is blocked, the precursor form accumulates in the stroma. Transport to and insertion into the thylakoid membrane appear to be dependent on the presequence and the presence of a ΔpH across the thylakoid membrane, and independent of ATP and a stromal extract, suggesting that this precursor follows a common import pathway with the 23K and 16K proteins of the OEC (figure 8), rather than that of pre-LHCP (Michl, Hulford, Robinson, Herrmann and Klösgen, unpublished data).

Figure 8 *Mechanisms of protein transport across the thylakoid membrane*

Two distinct groups of precursors can be seen with different requirements for translocation across the thylakoid membrane. One subgroup, containing 23K, 16K and CFo2, require a proton gradient only for translocation, whereas another, containing 33K and PC, require a stromal factor and ATP in addition to the proton gradient.


PC
CYTOSOL
33K
STROMA
23K
PC
16K
33K
STROMAL
FACTOR
ATP (ΔpH)
23K
16K
PC
ΔpH
33K
23K
16K
THYLAKOID
LUMEN

Cytochrome *f* is a chloroplast-encoded thylakoid membrane protein which is synthesised with a TTD (de Boer and Weisbeek, 1991) and contains an internal stop-transfer sequence. It contains a single membrane-spanning domain close to the C-terminus, with the N-terminal hydrophilic domain present in the thylakoid lumen (Willey *et al.*, 1984). The TTD, as for pre-$CF_0$2, directs the protein to the thylakoid network, probably following a similar pathway to that of thylakoid lumen proteins, and is also able to direct the protein to the *E. coli* cytoplasmic membrane (Rothstein *et al.*, 1985), again suggesting a common transport pathway with lumenal proteins. At least two pathways have therefore been identified for the insertion of proteins into the thylakoid membrane, one dependent on a TTD which is removed after insertion and the other dependent on regions of the mature protein.

1.3.4 Targeting of Proteins to the Chloroplast Envelope

Comparatively little is known about the mechanisms by which proteins are targeted to the envelope compartments, partly due to technical difficulties in isolating the individual envelope compartments (*i.e.* the outer and inner envelope membranes and the intermembrane space). Flügge and Wessel (1984) observed that one outer envelope membrane protein and two inner envelope membrane proteins were synthesised in a wheatgerm *in vitro* translation system as precursors of a higher molecular weight than that of the same proteins in the chloroplast envelope. One of the inner envelope proteins, the phosphate translocator, could be imported *in vitro* into isolated spinach chloroplasts where it inserted into the envelope membrane as the mature sized protein. This import was shown to be dependent on the presence of ATP and proteinaceous components on the surface of the chloroplast (Flügge *et al.*, 1989).

The maize *bt1* gene encodes a metabolite translocator protein located in the amyloplast membrane, which is synthesised as a larger precursor and can be imported into isolated chloroplasts *in vitro*, where it is processed to the mature size. The mature protein was found to be located in the inner envelope membrane (Li *et al.*, 1992) and the location of the targeting signals of this protein was investigated by the

construction of fusion proteins and analysis of their import into chloroplasts. A protein consisting of the presequence of rubisco SSU fused to the mature region of Bt1 was imported into chloroplasts and located in the inner envelope membrane, suggesting that the information for the targeting of Bt1 to the inner envelope membrane is contained within the mature protein and that the Bt1 presequence is a stroma-targeting signal.

Observations on the import of two outer envelope membrane proteins, one of 6.7 kDa (Salomon *et al.*, 1990) and one of 14 kDa (Li *et al.*, 1991) suggest that targeting of at least some outer envelope membrane proteins may involve a different pathway to that of transport into the chloroplast. These proteins do not contain a cleavable presequence and their targeting does not require ATP or a proteinaceous receptor. The insertion of the 14 kDa protein was shown to be specific for the chloroplast envelope membrane, but was not inhibited by synthetic peptide analogues of the SSU presequence, suggesting that the protein does not bind to the import receptor used by most chloroplast precursor proteins (Li *et al.*, 1991). The 6.7 kDa protein contains a hydrophobic core of 23 amino acids which could act as a stop-transfer sequence, analogous to that of proteins inserting into the E.R. membrane. However, Lubben *et al.* (1987) introduced a stop-transfer sequence into the mature region of a stromal protein and found that this was not sufficient to prevent targeting of the protein into the stroma, suggesting that a simple stop-transfer model comparable with that of E.R. targeting is unlikely and so chloroplast outer envelope membrane proteins may contain more complex targeting information than a simple stop-transfer domain.

1.3.5 Chloroplast Presequence Structure and Function

In many cases, the presequence of an imported chloroplast protein has been shown to be both necessary and sufficient for transport into the chloroplast, in particular by the use of fusion proteins whereby a chloroplast protein presequence is used to target a foreign protein to the chloroplast (section 1.3.6). The features of a

chloroplast presequence which determine its function are still poorly understood, with the very low conservation of primary structure between presequences suggesting either that higher order structure is the most important determinant or that presequences are unfolded structures with very little regular structure.

(a) Stromal Targeting Signals

Initial studies of the structural features of presequences concentrated on the presequence of rubisco SSU, as this was the first presequence whose sequence was known. A comparison of SSU presequences from different species led to the suggestion that three domains existed with distinct features (Schmidt and Mishkind, 1986). Domain I consisted of the N-terminal 12 to 25 residues and was positively-charged, rich in serine and threonine residues and hydrophilic. Domain II consisted of the next 11 to 18 amino acids in the centre of the presequence and was found to be highly conserved between SSU presequences, suggesting that this domain may be involved in receptor binding. Domain III was the C-terminal section of the presequence and was also rich in hydroxylated residues, with a conserved cysteine residue at the -1 postion (with the first residue of the mature protein being numbered +1). Pre-Fd was suggested to share the same import receptor as pre-SSU as its presequence contained a region homologous to SSU domain II, whereas pre-LHCP showed no homology to pre-SSU other than being serine and threonine rich and having an overall positive charge.

Karlin-Neumann and Tobin (1986) identified three blocks of homology separated by non-homologous regions between pre-LHCP and pre-SSU presequences and suggested that blocks I and II were important for binding and uptake and block III for SPP processing. Pre-SSU from *Chlamydomonas reinhardtii* was shown to contain blocks I and II only and was not processed to the mature size by higher plant chloroplasts. However, the Rieske iron-sulphur protein from spinach chloroplasts was found to have a presequence which did not correspond to the presequences already identified and did not contain the proposed homology blocks (Steppuhn *et al.*, 1987).

Robinson and Ellis (1985) showed that different residues may be important in different precursors by the incorporation of amino acid analogues into pre-SSU and pre-LHCP and investigation of their effects on import and processing of the precursors. The incorporation of analogues of proline, arginine and leucine markedly inhibited both import and processing, which could be due to overall conformational changes, particularly in the case of proline. Different amino acid analogues affected different precursors to varying extents, again providing evidence that overall structure may be more important than individual residues.

A detailed comparison of the structures of a much wider selection of chloroplast presequences has led to the conclusion that in general there are no regions of highly conserved amino acids between different precursors (figure 9) but that some overall structural features can be identified (von Heijne *et al.*, 1989). Three domains were identified, an N-terminal uncharged domain, a central domain with a low content of acidic residues and a C-terminal domain with a potential for forming an amphiphilic β-strand. Presequences have a net positive charge, due to an absence of acidic residues (Keegstra *et al.*, 1989), are rich in hydroxylated residues (20 to 35% of the total) and in small hydrophobic residues such as valine and alanine and have widely-varying lengths. The only highly conserved residues between presequences was methionine-alanine at the N-terminus and this may be a signal for removal of the N-terminal methionine (Pilon *et al.*, 1992). The lack of similarity could be due to precursors using different import receptors, but this is unlikely as no groups of precursors with similar presequences which may use the same receptor have been identified and it is likely that higher order structure is important (Keegstra *et al.*, 1989).

The function of the presequence in envelope membrane binding has been investigated by measuring the ability of 20 amino acid-long fragments of the pre-SSU presequence to interact with a monolayer of membrane lipids at an air/water interface. The peptides were found to be surface-active and to insert specifically into the lipid monolayers with a preference for chloroplast envelope lipids. Lipid-presequence

Figure 9 *Primary structures of the presequences of several stromal proteins*

The sequence of the targeting signals of rubisco SSU and rubisco activase from spinach and *Silene pratensis* ferredoxin in single letter code (taken from von Heijne *et al.*, 1989).

Spinach rubisco SSU
MASSVLSSAAVATVSRTPAQASMVAPFTGLKSTVGFPATKNDDITSLASNGGRVQC

Spinach rubisco activase
MATAVSTVGAATRAPLNLNGSSAGASVPTSGFLGSSLKKHTNVRFPSSSRTTSMTVKA

Silene pratensis ferredoxin
MASTLSTLSVSASLLPKQQPMVASSLPTNMGQALFGLKAGSRGRVTAM

interactions may therefore be involved in initial targeting steps, in addition to receptor-mediated specific binding to the chloroplast envelope (van't Hof *et al.*, 1991). Fragments of the pre-SSU presequence were also used in studies to test the effect of these peptides on the binding and translocation of pre-SSU, pre-LHCP, pre-Fd and pre-PC across the chloroplast envelope (Perry *et al.*, 1991). The peptides had the same effect on each precursor, with peptides corresponding to either end of the presequence having no effect on binding but inhibiting translocation and peptides from the middle of the presequence inhibiting binding. This suggests that the middle of the presequence is involved in binding and the ends in translocation, and that all of the precursors tested share components of the import apparatus. A role for the mature protein in targeting is implicated in work by Ko and Ko (1992), who synthesised chloroplast precursor proteins with truncated C-termini and import was found to be affected. C-terminal deletions of pre-33K caused a reduction in chloroplast import, aberrant and reduced processing, and incorrect and inefficient thylakoid targeting, whereas C-terminal deletions of pre-ferredoxin-NADPH reductase (pre-FNR) had no effect on stromal targeting but caused a decrease in thylakoid targeting. Pre-SSU C-terminal deletions caused a severe reduction in both binding and import but deletions of rubisco activase had little effect and it was concluded that the overall structure of the entire protein may be involved in targeting in some cases.

Hydrophobic moment analysis was used by Theg and Geske (1992) to predict the secondary structure of the presequence of the precursor to the γ-subunit of the chloroplast ATP synthase from *Chlamydomonas reinhardtii*, which was considered a good model for higher plant precursors as it was able to inhibit the import of pea pre-SSU into pea chloroplasts. An amphiphilic β-sheet structure was predicted; however, this conflicted with circular dichroism (CD) data suggesting that the presequence adopted a random coil structure even in the presence of detergent micelles. Pilon *et al.* (1992) analysed the full precursor, mature protein and chemically-synthesised presequence of pre-Fd using circular dichroism, tryptophan fluorescence quenching and protease accessibility and also came to the conclusion that the precursor has a low

content of secondary and tertiary structure due to both the mature protein and the presequence, similar to that found in unfolded proteins. The presequence was seen to be flexible and to exist in different conformations in different environments, and the mature protein had little effect on the conformation of the presequence, behaving as a functionally independent unit. This was taken to suggest that the presequence undergoes a conformational change, by interacting with membrane lipids for example, which leads to the formation of secondary structure which can specifically interact with the import apparatus. The function of cytosolic factors for the import of some precursor proteins into chloroplasts may be to aid unfolding of the precursor to allow interaction with the membrane components, whereas the more flexible pre-Fd shows no requirement for a cytosolic factor. Von Heijne and Nishikawa (1991) concluded from the overall lack of secondary structure that could be predicted within presequences that chloroplast presequences may be designed to be flexible peptides with very little secondary or tertiary structure *ie* random coils, and that targeting specificity was maintained by sequential binding of the precursors to a series of chaperones along the import pathway. The involvement of a series of chaperones in protein import into the mitochondrial matrix has been demonstrated by Manning-Krieg *et al.* (1991) and a similar process can be envisaged in import into chloroplasts.

(b) Thylakoid Targeting Signals

A comparison of the thylakoid targeting signals which make up the C-terminal domain of some nuclear-encoded thylakoid proteins and the presequence of some chloroplast-encoded thylakoid proteins reveals several common features (Keegstra *et al.*, 1989; von Heijne *et al.*, 1989). A short 'n' domain at the N-terminus with a net positive charge is followed by a central 'h' region of 14 - 22 amino acids containing a stretch of 7 - 15 hydrophobic amino acids. A 5 - 6 residue 'c' domain immediately before the final cleavage site is rich in small hydrophobic amino acids and is usually preceded by a turn-inducing residue (proline, glycine, serine, aspartate or asparagine).

Small, hydrophobic residues, typically alanine, are found at the -1 and -3 positions, where the first residue of the mature protein is numbered +1 (figure 10).

TTDs have many features in common with signal peptides directing the export of proteins from the bacterial cytoplasm, suggesting that a similar transport mechanism may operate in both situations. Both contain a positively-charged N-terminus, a central hydrophobic domain and small hydrophobic residues at the -3 and -1 positions. The amino acid composition of the hydrophobic region of TTDs, however, differs significantly from that of signal peptides. The similarities are sufficient that the chloroplast 33K precursor is exported by the bacterial export machinery and processed to the mature size by the bacterial signal peptidase when the protein is expressed in *Escherichia coli* (Meadows and Robinson, 1991).

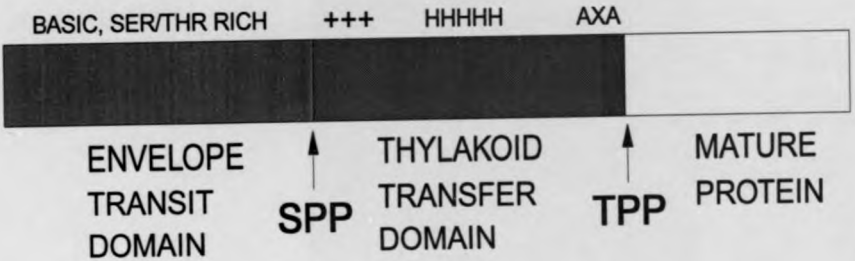
Thylakoid lumen targeting signals and bacterial export signals are also similar to the signals targeting mitochondrial proteins to the mitochondrial intermembrane space (IMS). Some nuclear-encoded IMS proteins are also synthesised with a bi-partite presequence which directs them initially to the mitochondrial matrix, where the matrix-targeting domain is removed, and the IMS targeting signal then directs translocation back across the inner membrane to the IMS. The overall structure of the IMS targeting domain is similar to that of a TTD although they do not always follow the alanine-X-alanine rule prior to the cleavage site (Dalbey and von Heijne, 1992).

1.3.6 Targeting of Foreign Proteins into Chloroplasts

The ability of a chloroplast protein presequence to target a foreign protein into chloroplasts demonstrates that the presequence contains all of the information required for targeting. This was first shown by van den Broeck *et al.* (1985) who created a chimaeric gene consisting of the section of the gene encoding the presequence of pre-SSU linked to the neomycin phosphotransferase II (*nptII*) gene. The construct was expressed in tobacco plants and the *nptII* gene product was found as the processed form in the chloroplast stroma. The same protein was synthesised in

Figure 10 *Structural features of a thylakoid lumen protein presequence*

The presequence contains two domains, an envelope transit domain responsible for targeting across the chloroplast envelope and a thylakoid transfer domain which directs targeting to the thylakoid lumen. The envelope transit domain has an overall positive charge and is rich in hydroxylated and deficient in acidic residues. The thylakoid transfer domain has a positively charged N-terminus, a central hydrophobic core region (HHHHH) and small hydrophobic residues at the -1 and -3 positions.


BASIC, SER/THR RICH
+++
HHHHH
AXA
ENVELOPE TRANSIT DOMAIN
SPP
THYLAKOID TRANSFER DOMAIN
TPP
MATURE PROTEIN

E. coli and incubated with isolated intact pea chloroplasts, with the activity again being localised to the stroma.

Both Wasmann *et al.* (1986) and Kuntz *et al.* (1986) used fusion proteins consisting of the pre-SSU presequence, or presequence and 23 amino acids of the mature protein, linked to nptII to investigate the effect of the N-terminus of the mature protein on import efficiency. Wasmann *et al.* assayed these proteins in an *in vitro* chloroplast import system and found that the presequence with some of the mature SSU protein was capable of directing import of nptII into isolated chloroplasts with a much greater efficiency than the presequence alone. In contrast, Kuntz *et al.* found that *in vivo* in transformed tobacco plants the construct containing no mature protein was the most efficient at directing accumulation of nptII in chloroplasts. This was proposed to be due to more rapid degradation *in vivo* of the larger fusion protein. In similar experiments by Lubben *et al.* (1989) in which brome mosaic virus coat protein was used as a reporter protein and linked to the presequence and varying lengths of mature protein of pre-SSU, the presequence alone and the presequence with a small section of mature protein were seen to direct import into isolated chloroplasts. However, the presequence along with the majority of the mature protein failed to direct import of the foreign protein, possibly because the protein folded into an import-incompetent conformation very quickly after synthesis. Chimaeric genes encoding the presequence and varying lengths of mature protein of pre-LHCP and *E. coli* β-glucuronidase were used to transform tobacco (Kavanagh *et al.*, 1988) and more than 16 amino acids of mature protein were found to be required for accumulation within chloroplasts, in contrast to the results obtained for pre-SSU.

Targeting of foreign proteins has also been achieved using thylakoid lumen protein presequences, in particular with the pre-33K presequence, with a variety of final locations of the targeted proteins seen. Dihydrofolate reductase was efficiently targeted by the pre-33K presequence to the thylakoid lumen *in vitro*, with proteolytic removal of the presequence in the lumen (Meadows *et al.*, 1989) whereas glycollate oxidase was targeted only as far as the stroma, where the ETD was removed leaving

an intermediate sized product (Ko and Cashmore, 1989). The pre-33K presequence directed ricin A chain into chloroplasts where it was localised partly in the stroma and partly bound to the stromal side of the thylakoid membrane (Roberts *et al.*, 1991). The entire presequence was removed, indicating that the protein must have been targeted at least partially across the thylakoid membrane.

The problem of differing results for the targeting of proteins *in vivo* and *in vitro* was addressed by de Boer *et al.* (1991) by assaying the targeting of a foreign protein into chloroplasts both *in vivo* and *in vitro* by various presequences. β-lactamase was fused to the C-terminus of:

(a) Fd presequence

(b) PC presequence

(c) PC presequence containing an 11 amino acid deletion in the TTD

(d) Fd presequence plus PC TTD

(e) PC full precursor

Both *in vivo* and *in vitro*, (a) and (c) were transported into the stroma and cleaved by SPP as expected. *In vitro*, (b) and (d) were also located in the stroma and cleaved by SPP to the intermediate size only but *in vivo* were targeted correctly to the thylakoid lumen and processed to the mature size. (e) was located both *in vivo* and *in vitro* in the thylakoids but not protease-protected, despite being processed to the mature size, suggesting that the PC part may translocate across the membrane but the β-lactamase part may remain in the membrane. This study demonstrates the problems of applying results obtained in an *in vitro* situation to events occuring *in vivo* and demonstrates that import of proteins into isolated chloroplasts may not always reflect their location in chloroplasts *in vivo*.

1.3.7 Specificity of Chloroplast Protein Targeting

Plant cells, containing both chloroplasts and mitochondria (as well as other organelles), have the problem of sorting proteins synthesised in the cytosol to the required organelle. This process appears in general to be highly specific for each organelle, but there are a few examples of mis-sorting of proteins.

Antibodies raised against rubisco SSU from *Chlamydomonas reinhardtii* were used to determine the localisation of SSU in the alga *Ochromonas danica* by *in situ* hybridisation (Lacoste-Royal and Gibbs, 1985). Both the chloroplast and mitochondria of *O. danica* were labelled, whereas only the chloroplast was labelled in *C. reinhardtii*. Antibodies against rubisco LSU did not label mitochondria in *O. danica* or *C. reinhardtii*. As it was not known whether SSU in *O. danica* is nuclear- or chloroplast-encoded, it is difficult to draw conclusions from this data regarding mis-targeting of SSU to mitochondria. The authors suggested that this explanation is unlikely as the *O. danica* chloroplast is completely enclosed by rough endoplasmic reticulum (RER) at which nuclear-encoded chloroplast proteins are thought to be synthesised and transported co-translationally into the ER lumen. Proteins are transported from the ER lumen to the chloroplast envelope via transport vesicles. Chloroplast proteins may thus never come into contact with mitochondria and it was instead proposed that the DNA sequence encoding SSU had migrated to the mitochondria from the nucleus or chloroplast and was expressed within the organelle.

Presequences of chloroplast proteins from *Chlamydomonas reinhardtii* have structural features which are more similar to mitochondrial presequences in that they have a high arginine content and a tendency to form an amphiphilic α-helix (von Heijne, 1991). The presequence of pre-SSU from *C. reinhardtii* is able to direct the import of DHFR and the mitochondrial cytochrome oxidase subunit IV (cox IV) lacking its presequence into yeast mitochondria, although with a lower efficiency than an authentic mitochondrial presequence (Hurt *et al.*, 1986), implying that *C. reinhardtii* must employ different mechanisms for ensuring specificity of targeting to mitochondria than higher plants and yeast and to chloroplasts than higher plants. Mis-

targeting in higher plants has also been demonstrated by Huang *et al.* (1990) by the use of a fusion protein consisting of the presequence of yeast mitochondrial cytochrome oxidase subunit Va linked to a chloramphenicol acetyltransferase reporter protein. After expression in tobacco plants, the fusion protein was found in mitochondria and chloroplasts at approximately equal levels and processed in both cases by removal of the presequence.

Despite these reports of mis-targeting between chloroplasts and mitochondria, proteins are in general thought to be targeted very specifically to one or other organelle, with mis-targeting being the exception rather than the rule. For example, Smeekens *et al.* (1987) fused the presequences from Fd and PC in front of yeast mitochondrial manganese superoxide dismutase lacking its mitochondrial presequence and the fusion protein was targeted exclusively to chloroplasts and not mitochondria in *in vitro* import experiments, even though the targeted protein is normally located in mitochondria. Whelan *et al.* (1990) found that the precursor of the β-subunit of the mitochondrial F_1 ATPase could be imported *in vitro* into spinach leaf mitochondria but not chloroplasts, and that the chloroplast pre-33K protein could be imported into chloroplasts but not mitochondria. These results demonstrate the specificity of targeting between mitochondria and chloroplasts which enables them to import an organelle-specific set of proteins from the cytosol.

1.3.8 Proteolytic Processing of Imported Chloroplast Proteins

After the import of proteins into the chloroplast stroma, the stromal targeting signal is removed by the action of a stromal processing peptidase (SPP). Thylakoid lumen proteins are further targeted across the thylakoid membrane into the lumen, where the TTD is removed by a thylakoidal processing peptidase (TPP).

(a) Stromal Processing Peptidase

Chua and Schmidt in 1978 showed that pea and spinach SSUs were synthesised as precursors and were processed to the mature size after import into isolated intact chloroplasts. The processing activity was shown to be located in the stroma by Smith and Ellis (1979) who found that a stromal extract could process pre-SSU to the mature size. SPP was purified ~350-fold by Robinson and Ellis (1984a) from pea chloroplasts and the elution profile of activity from a Sephacryl S-300 and DEAE-Sephacel column suggested that a single protein was responsible for the cleavage of pre-SSU, pre-PC and pre-FNR. The specificity of the enzyme for the chloroplast precursor proteins was demonstrated by its inability to cleave any rotavirus mRNA translation products. The SPP activity had a pH optimum of 8-9, was not ATP-dependent and was inhibited by 1,10-phenanthroline and EDTA but not by EGTA, phenylmethylsulphonylfluoride (PMSF) or iodoacetate, suggesting that it is a metalloprotease. Calibrated gel filtration suggested that the enzyme had a MW of ~180,000. An activity was identified by Abad *et al.* (1989) which processed pre-LHCP in an organelle-free reaction and co-eluted from a gel filtration column with the activity cleaving pre-SSU, suggesting that SPP cleaves both of these precursor proteins. Truncated precursors with C-terminal deletions were still processed and the processing event must therefore remove residues from the N-terminus of the precursor. SPP activity from pea chloroplasts was eventually purified by Oblong and Lamppa (1992b) using affinity chromatography on immobilised, *E. coli*-expressed pre-LHCP to produce a doublet of proteins upon SDS-polyacrylamide gel electrophoresis with MWs of 145,000 and 143,000. These two proteins co-eluted at every step throughout the purification but could never be identified as a complex by native gel electrophoresis. Antibodies were raised against the two proteins, which were found to be immunologically related, although the relationship of the two proteins to each other is as yet unknown; the proteins could be isoenzymes, related subunits of an enzyme complex or the smaller could be a degradation product formed during the purification. The purified proteins were able to process pre-LHCP, pre-

SSU and pre-acyl carrier protein (ACP) to their mature sizes, confirming that the same enzyme is involved in the processing of many imported precursor proteins, although it is not known whether they will also process precursors to thylakoid lumen proteins.

There have been several reports of the processing of stromal targeting presequences at two sites, involving either a two-step sequential cleavage or cleavage at one of two alternative sites in the precursor. The best characterised example of the latter is pre-LHCP, which is cleaved upon import into chloroplasts at a primary or secondary site to give rise to a heterogeneous population of molecules of either 26 or 25 kDa. Only cleavage at the secondary site is performed by SPP and seen in an organelle-free assay, with primary site cleavage due to another protease which has yet to be identified (Clark and Lamppa, 1991). A similar situation is seen in the processing of pre-carbonic anhydrase, which is processed at two sites to produce subunits of two different sizes, with only one of the processing events due to SPP (Johansson and Forsman, 1992).

Processing of pea pre-SSU occurs in two steps (Robinson and Ellis, 1984b) with the processing intermediate appearing at early time points in a time course analysis of cleavage. The activities responsible for the two steps could not be separated by column chromatography, suggesting that SPP is responsible for both reactions. Pre-SSU from *Chlamydomonas reinhardtii* can be imported into pea chloroplasts, but is processed to an intermediate size only (Mishkind *et al.*, 1985), suggesting that processing of pre-SSU in *Chlamydomonas* also occurs in two steps but that the processing mechanisms in the alga may be different to that in higher plants. The *Chlamydomonas reinhardtii* chloroplast ribosomal protein L-18 is also processed in two steps, but by an apparently different mechanism to pre-SSU (Schmidt *et al.*, 1985). The first, very rapid, processing step occurs shortly after or during import and is probably due to SPP. The second step occurs during ribosome assembly and is slow, requiring chloroplast protein synthesis and ribosomal proteins and is thus unlikely to be catalysed by SPP (Liu *et al.*, 1988).

Mutagenesis studies and sequence comparisons have revealed some of the features required for recognition of a processing site by SPP, although there is very little sequence homology between precursors and nothing is yet known about the reaction mechanism of SPP. The incorporation of amino acid analogues into the pre-SSU presequence (Robinson and Ellis, 1985) implied that overall structural features of the presequence as well as the residues around the processing site were important for the cleavage of this precursor, and this has been found to be true for many different precursor proteins. In the case of pre-LHCP, the residues immediately surrounding the processing site were shown to determine the efficiency of cleavage (Clark and Lamppa, 1991), although other features probably specify the site of cleavage. Karlin-Neumann and Tobin (1986) identified a block of homology between pre-LHCP and pre-SSU cleavage sites which contained glycine residues known to be disrupters of secondary structure and suggested that the region around the cleavage site was kept in a random coil conformation to render it susceptible to proteolytic cleavage. However, deletion mutagenesis of pre-SSU (Wasmann *et al.*, 1987) showed that this homology block was not essential for cleavage but that a three residue sequence (Ile-Thr-Ser) close to the C-terminal region of the presequence was required for correct processing. Sequence analysis has identified some general features of cleavage sites; the region next to the cleavage site often has the potential to form a β-strand, turn-inducing residues are absent between the -3 and +1 positions, and alanine is enriched at the cleavage site (von Heijne *et al.*, 1989). A more detailed analysis of 32 stromal targeting signals from a wide variety of precursors yielded a weakly-conserved consensus sequence around the processing site of (Val/Ile)-X-(Ala/Cys) ↓ Ala, with the arrow marking the site of cleavage (Gavel and von Heijne, 1990) and the -6 to -10 positions were enriched in arginine residues. Around 30% of sequences were a perfect match to the consensus, but there were many exceptions implying yet again that more complex features are involved in specifying the cleavage site.

(b) Thylakoidal Processing Peptidase

The thylakoidal processing peptidase (TPP) responsible for removing the TTD from imported chloroplast proteins was first described by Hageman *et al.* (1986). An artificial intermediate form of plastocyanin was used to assay processing activity, which could be detected by incubation with Triton X-100-disrupted thylakoids but not by incubation with intact thylakoids. TPP was partially purified by hydroxylapatite and DEAE-Sephacel chromatography (Kirwin *et al.*, 1987) and properties of the enzyme were found to include a pH optimum of 6.5-7, activation by the chelating agents EDTA and EGTA, with no inhibitors for the enzyme yet described. TPP also processed precursors to the 33K, 23K and 16K proteins to the mature size, suggesting a general role in thylakoid lumen protein maturation (James *et al.*, 1989). TPP is tightly bound to the thylakoid membrane with its active site on the lumenal face, is not associated with any of the major thylakoid membrane complexes and is located exclusively in non-appressed lamellae of the thylakoid network (Kirwin *et al.*, 1988).

TTDs and bacterial leader peptides are similar in structure and so the specificities of TPP and *E. coli* leader peptidase (LEP) were compared (Halpin *et al.*, 1989). LEP cleaved pre-23K and pre-33K, with cleavage taking place at the correct site at least for pre-23K, and both eukaryotic and prokaryotic leader peptides were removed by TPP. A synthetic leader peptide also inhibited both LEP and TPP, and the reaction specificities of the two enzymes therefore considered to be identical. Site-directed mutagenesis of the -3 and -1 positions of the pre-33K presequence showed that the requirements for cleavage of a precursor by TPP are more restrictive than for LEP or eukaryotic signal peptidase (SP; Shackleton and Robinson, 1991). For example, a leucine residue is tolerated by both LEP and SP at the -3 position (Fikes *et al.*, 1990; von Heijne, 1983) but not by TPP and an alanine residue at the -1 position appears to be critical for TPP cleavage. A method for predicting TPP cleavage sites was developed by Howe and Wallace (1990) which accurately predicted cyanobacterial and higher plant nuclear-encoded precursor TPP cleavage sites but not

chloroplast-encoded thylakoid protein TPP cleavage sites, perhaps implying that a different protease is responsible for the maturation of these proteins.

TPP is now thought to be encoded by the chloroplast genome, with two lines of evidence from chloroplast genome mutants. The first is the tobacco *lutescens-1* mutant (Chia and Arntzen, 1986) which accumulates intermediate sized 23K and 33K which are located on the inner surface of the thylakoid membrane but not associated with PS II complexes, which are in fact absent in this mutant. The second is the *Oenothora* mutant *pm7* (Johnson *et al.*, 1991) in which cytochrome *f*, 23K and 16K are found as intermediates, which also provides evidence that the same protease may be responsible for maturation of nuclear- and chloroplast-encoded thylakoid proteins as cytochrome *f* is chloroplast-encoded and 23K and 16K are nuclear-encoded.

1.3.9 Applications of Chloroplast Protein Targeting

The most advanced report of the application of chloroplast protein targeting is in herbicide resistance. Glyphosate is a herbicide which inhibits the activity of 5-enolpyruvylshikimate-3-phosphate ($E^{r}SP$) synthase, a nuclear-encoded chloroplast enzyme which is imported into the stroma where it is involved in the shikimate pathway. An *E. coli* gene encoding a mutant EPSP synthase which is resistant to glyphosate was fused downstream of the cDNA encoding the presequence of EPSP synthase from *Petunia hybrida*, and the fusion protein was synthesised *in vitro* and could be imported into isolated chloroplasts to produce the mature-sized, glyphosate-resistant enzyme (della-Cioppa *et al.*, 1987). The inhibition of wild type EPSP synthase activity by glyphosate was due to inhibition of import into chloroplasts as a direct result of glyphosate binding to the pre-EPSP synthase-shikimate-3-phosphate complex which suggests that glyphosate acts by preventing unfolding of the precursor, which may be required for translocation across the envelope membranes (della-Cioppa and Kishore, 1988).

Herminghaus *et al.* (1991) proposed an approach towards optimising plant secondary metabolite pathways in which genes are introduced into a plant which

normally encode rate-limiting steps in a pathway, thereby increasing their synthesis and activity to enable the rate of the overall pathway to increase. Lysine decarboxylase (LDC) catalyses the first step in the conversion of lysine to alkaloids, the decarboxylation of lysine to cadaverine, and in general has a low activity in higher plants. As lysine biosynthesis occurs in the chloroplast, the LDC activity was required in the chloroplast where a large pool of substrate would be available. A bacterial *ldc* gene was cloned downstream of the promoter and presequence coding region of potato SSU and introduced into tobacco plants, where the gene was expressed and LDC activity located in chloroplasts due to the presence of the SSU presequence. The increased activity in the transformed plants was measured by the large increase in cadaverine levels compared with the wild type plants. It remains to be seen whether this approach may be used to increase yields of a complete pathway rather than accumulating intermediates.

1.4 Transport of Proteins Into and Within Mitochondria

Research into the mechanisms of protein targeting into mitochondria is more advanced than that of chloroplasts and some similarities can be seen between the overall features of the two processes. The mitochondrion consists of two membranes, the outer and inner membrane, between which is the intermembrane space, and the inner membrane encloses the mitochondrial matrix. The mitochondrion, like the chloroplast, contains its own genome and so can synthesise a subset of proteins required in the mitochondrion, but the majority of mitochondrial proteins are nuclear-encoded and so are imported into the organelle after synthesis in the cytosol, often by means of an N-terminal presequence which is removed after import, although there are many exceptions.

1.4.1 Conformation of Translocated Proteins

Much evidence has now been presented that a protein must be in an unfolded conformation in order to cross the mitochondrial membrane, with initial work performed by Eilers and Schatz (1986) using a fusion protein of a mitochondrial presequence linked to DHFR. In the absence of methotrexate this protein could be imported into mitochondria but the binding of methotrexate to the DHFR moiety led to the stabilisation of the DHFR structure and inhibition of import, showing that DHFR must be at least partially unfolded for translocation across the membrane. Similar experiments by Chen and Douglas (1987b) confirmed the validity of this approach, by replacing the C-terminus of the precursor to the β-subunit of the F_1ATPase (pre-$F_1\beta$) with part of copper metallothionein. In the absence of copper, the fusion protein could be imported, but copper prevented import by binding to the metallothionein and preventing it from unfolding. A hybrid protein consisting of the N-terminal third of cytochrome b_2 fused to DHFR accumulated at contact sites between the inner and outer membranes in the presence of methotrexate, causing the inhibition of import of authentic mitochondrial precursor proteins (Wienhues *et al.*, 1991). The use of varying lengths of the portions of cytochrome b_2 indicated that around 50 amino acids were sufficient to span both membranes, providing evidence for the linear translocation of unfolded polypeptide chains. *In vivo*, however, methotrexate did not affect the import of DHFR hybrid proteins, which was taken as evidence that import occurs co-translationally *in vivo* (Fujiki and Verner, 1993). Further evidence for this was that after preventing import *in vivo* to allow a build up of precursor proteins in the cytosol, removing the import block showed that different precursors displayed varying efficiencies for post-translational import. However, this could reflect the folding of precursors in the cytosol into conformations which were incompatible with import. A different approach to the question of whether unfolding is required for import was that of site-directed mutagenesis of a precursor which caused destabilisation of the protein structure (Vestweber and Schatz, 1988).

Destabilising mutations caused an increase in the rate and efficiency of import, thus suggesting that stability of tertiary structure is a disadvantage in the import reaction.

Soluble cytosolic components have been implicated in the maintenance of precursor proteins in an import-competent conformation. Chen and Douglas (1987a) reported that import of pre-$F_1\beta$ required soluble components from rabbit reticulocyte lysate and Murakami and Mori (1990) subsequently purified a 50 kDa presequence binding factor (PBF) from rabbit reticulocyte lysate which stimulated the import of precursors into rat liver mitochondria. Yeast hsp70 further stimulated import, but alone had little effect, suggesting cooperation between PBF and hsp70 to hold precursors in an import-competent conformation. Cytosolic factors are not required for the import of all precursor proteins, and so PBF-dependent and -independent pathways are thought to exist (Becker *et al.*, 1992; Murakami *et al.*, 1992). The unfolding of precursors prior to translocation was shown to be dependent on nucleotide triphosphates (NTPs), with the level of NTP required depending on the mature protein (Pfanner *et al.*, 1987). This could reflect a requirement for NTPs in the transfer of proteins from cytosolic binding factors to receptors in the mitochondrial membrane. A cytosolic factor from rat liver which could stimulate the import of precursor proteins into isolated mitochondria was purified by Hachiya *et al.* (1993). This factor was shown to unfold mitochondrial precursor proteins in an ATP-dependent reaction, thus confering import-competence.

1.4.2 Import of Proteins Into the Mitochondrial Matrix

The existence of proteinaceous receptors on the surface of mitochondria involved in the import of precursor proteins was first demonstrated by protease-treatment of isolated intact *Neurospora crassa* mitochondria, which inhibited the ability of the mitochondria to bind and import several mitochondrial precursor proteins (Zwizinski *et al.*, 1984). Antibodies against yeast mitochondrial surface proteins caused inhibition of import, also suggesting that a surface protein was involved in import (Ohba and Schatz, 1987). Two receptors for the import of proteins

have now been identified (Segui-Real *et al.*, 1992), a 19 kDa outer membrane protein (MOM19 in *N. crassa*) which is responsible for the recognition of most precursor proteins containing a cleavable presequence (Schneider *et al.*, 1991; Moczko *et al.*, 1993) and a 72 kDa protein (MOM72) which recognises a subset of imported proteins, including the ADP/ATP carrier which does not have a cleavable presequence but has targeting information located in small sections of sequence along its length. The yeast MOM72 homologue (MAS70) accelerated the import of many but not all precursor proteins *in vitro*, and removal of MAS70 inhibited binding to the surface of the mitochondria, indicating that it is involved in a very early step in protein import (Hines *et al.*, 1990). However, *in vivo* experiments in yeast cells showed MAS70 to accelerate the import of all precursor proteins tested (Hines and Schatz, 1993) and it was concluded that MAS70 is the import receptor for most, if not all, yeast mitochondrial precursor proteins but that the step involving MAS70 is not always rate-limiting in experiments with isolated yeast mitochondria. A third protein is also thought to function as an import receptor in yeast, a 32 kDa outer membrane protein which was cloned by Murakami *et al.* (1990) and the nucleotide sequence revealed that it was similar to mammalian phosphate translocators, although the significance of this is not clear. Yeast mutants defective in this protein indicated that it can function in the import of some but not all precursors. As none of the receptor proteins are essential for cell viability, they are thought to be functionally redundant, with overlapping specificities for precursor proteins (Baker and Schatz, 1991).

Pfaller *et al.* (1988) hypothesised that after the binding of precursor proteins to specific receptors in the outer membrane, all precursors are transferred to a general insertion protein component (GIP) in the outer membrane from which they are translocated across the membrane. Evidence for the hypothesis was provided as the precursor of porin, an outer membrane protein, competed for import with proteins from every mitochondrial compartment, despite using different receptor proteins, suggesting that competition occurred at a later step in the pathway. The first component of the GIP to be identified was a 42 kDa yeast outer membrane protein

discovered by cross-linking of a chimaeric protein stuck in the import site (Westweber *et al.*, 1989) termed ISP42. Other components in *N. crassa* were identified by cross-linking of translocation intermediates to the complex and co-immunoprecipitation (Söllner *et al.*, 1992). The precursor was cross-linked to the MOM19 and MOM72 receptors, the *N. crassa* homologue of ISP42 (MOM38), and three novel outer membrane proteins, MOM7, MOM8 and MOM30, which formed the GIP site along with MOM38. The isolated yeast import complex also contained MOM22, of unknown function (Moczo *et al.*, 1992).

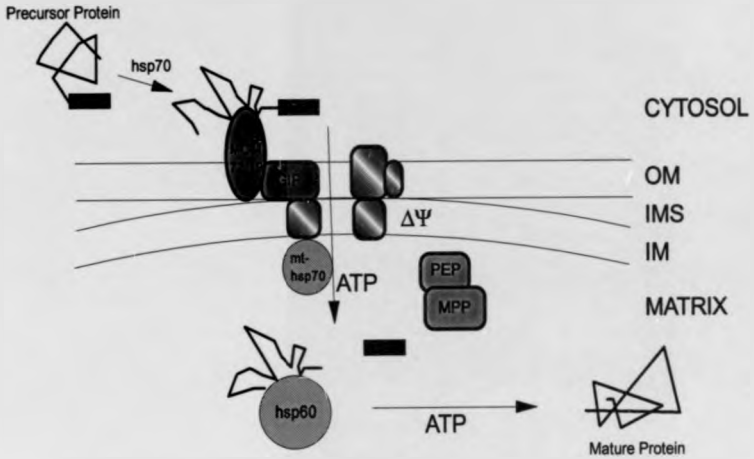
Identification of components of the mitochondrial inner membrane translocation system has proved to be difficult, but recently a 45 kDa protein at the yeast inner membrane import site was identified (Scherer *et al.*, 1992; Horst *et al.*, 1993) and found to be identical to a previously described 49 kDa protein involved in import which is essential for yeast viability (Maarse *et al.*, 1992).

The transport of proteins across the inner membrane into the matrix requires an energised inner membrane for the N-terminus of the mature protein to reach the matrix, from which point there is no further requirement for a pmf for completion of translocation. The import of various precursors showed different sensitivities to the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), with the variation depending only on the presequence (Martin *et al.*, 1991). A membrane potential ($\Delta\Psi$) was shown to be the component of the pmf required for import, with the ΔpH having no effect, in contrast with the situation in chloroplasts (section 1.3.2). The driving force for completion of translocation into the matrix is the binding of the N-terminus of the precursor to the mitochondrial chaperone hsp70 as it emerges from the inner membrane. The precursor is thought to be pulled through the membrane by interactions with hsp70, with the release of hsp70 requiring ATP hydrolysis (Neupert *et al.*, 1990). Some precursors may then bind to a mitochondrial hsp60 to allow their correct folding and assembly (Manning-Krieg *et al.*, 1991; figure 11).

The nature of the interaction between the protein translocation machinery in the inner and outer membranes has been the subject of some debate. The inner and

Figure 11 Transport of proteins across the mitochondrial membranes

Hypothetical model for the import of precursor proteins into mitochondria. Mitochondrial precursor proteins are recognised by receptor proteins in the mitochondrial outer membrane and inserted into the membrane at the general insertion site. Precursors are translocated through a proteinaceous pore in the membrane using a $\Delta\Psi$-dependent mechanism. The mitochondrial hsp70 binds to the precursor proteins emerging from the membrane and, after removal of the presequence, the mature protein is folded in association with hsp60.


Precursor Protein
hsp70
CYTOSOL
OM
IMS
IM
MATRIX
ΔΨ
mt-hsp70
ATP
PEP
MPP
hsp60
ATP
Mature Protein

outer membranes are known to cooperate in import into the matrix as translocation intermediates spanning both membranes have been found (Baker and Schatz, 1991). Current opinion is that the translocation machinery in each membrane is mobile in the plane of the membrane and does not form a permanent channel, but that the outer and inner membrane machinery are usually used together (Pfanner *et al.*, 1992). Mitochondrial outer membrane vesicles were shown to be capable of importing certain proteins in the absence of inner membrane, suggesting that the outer membrane translocation machinery can function independently of the inner membrane (Mayer *et al.*, 1993). Evidence for this model is provided by the formation of translocation intermediates by either depleting the amount of ATP in the import reaction or depleting the $\Delta\Psi$ across the inner membrane. These intermediates were membrane bound but exposed to the intermembrane space, as they were protease-accessible after disruption of the outer membrane. The intermediates could be chased into the matrix by the restoration of the appropriate import conditions, showing that they were genuine productive intermediates (Rassow and Pfanner, 1991; Hwang *et al.*, 1991; Jascur *et al.*, 1992). Segui-Real *et al.* (1993) showed that fusion proteins consisting of the N-terminus of pre-$F_1\beta$ (a matrix-located protein) linked to cytochrome *c* haem lyase (CCHL; an IMS protein with its targeting information in the mature protein) could be imported into the IMS in the absence of $\Delta\Psi$ using the CCHL pathway, or into the matrix in the presence of $\Delta\Psi$ using the $F_1\beta$ pathway. The protein in the IMS could be chased into the matrix upon restoration of the $\Delta\Psi$, demonstrating that the translocation systems in both the outer and inner membranes could function independently.

1.4.3 Routing of Proteins to the Sub-compartments of Mitochondria

The intermembrane space proteins cytochrome b_2 and cytochrome c_1 were originally reported to be imported by a 'conservative sorting' mechanism whereby the precursor proteins were imported into the matrix, processed to the intermediate size by MPP/PEP and then re-exported to the intermembrane space and processed to the

mature size. If MPP/PEP was inhibited, the precursor accumulated in a protease-protected compartment, deduced to be the matrix by fractionation of the mitochondria using osmotic shock (Hartl *et al.*, 1987). Koll *et al.* (1992) proposed that the export of intermembrane space proteins from the matrix was mediated by their interaction with matrix-located hsp60. The interaction of cytochrome b_2 with hsp60 was shown to prevent its folding and the intermembrane space targeting domain of the presequence suggested to inhibit the ATP-dependent release of the cytochrome from hsp60, thus allowing it to interact with the inner membrane export machinery. However, Glick *et al.* (1992) presented evidence for a 'stop-transfer' model for the import of these two proteins, as import intermediates were found only outside the inner membrane and both presequences were able to direct import of DHFR into the intermembrane space in the absence of ATP, suggesting that translocation across the inner membrane did not occur. Yeast mutants defective in hsp60 function were found to import and process both cytochrome b_2 and cytochrome c_1 correctly, which is consistent with this hypothesis (Hallberg *et al.*, 1993). Cytochrome c_1 was imported normally after depletion of matrix ATP, as was the adenine nucleotide translocator, an inner membrane protein (Wachter *et al.*, 1992) and cytochrome *c* haem lyase (Lill *et al.*, 1992), suggesting that this may be a common pathway for import into compartments other than the matrix. The precursor to the inner membrane protein cytochrome *c* oxidase subunit Va requires ATP and a $\Delta\Psi$ for correct localisation, but import is independent of protease-sensitive components on the mitochondrial surface (Miller and Cumsky, 1991) and apocytochrome *c* can also translocate across the outer membrane in a receptor-independent manner, with its interaction with cytochrome *c* haem lyase in the outer membrane aiding translocation (Jordi *et al.*, 1992). Therefore, it appears that there are multiple mechanisms for transport to the intermembrane space and inner membrane (figure 12). The insertion of an outer membrane protein (OMP70) was investigated by McBride *et al.* (1992) by synthesising a hybrid protein consisting of the N-terminus of the outer membrane protein, which did not have a cleavable presequence, to DHFR and import was found to be dependent on ATP and a

Figure 12 *Transport of proteins into the mitochondrial intermembrane space*

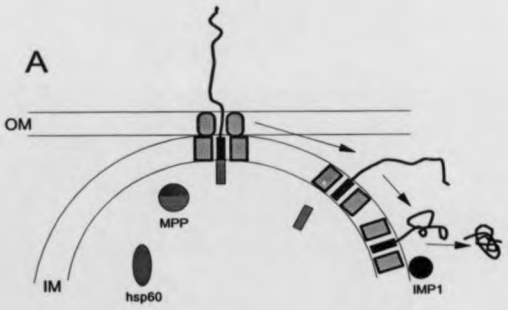
Two hypotheses for the mechanism of protein targeting to the mitochondrial intermembrane space (Adapted from Glick *et al.*, 1992).

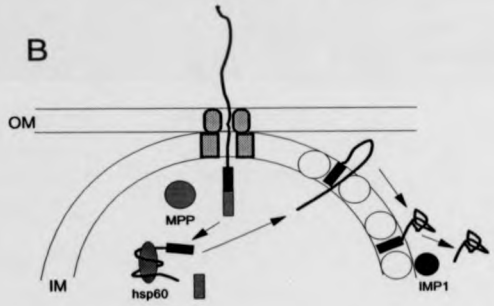
Panel A

Stop transfer mechanism. The precursor inserts into the membrane but the intermembrane space targeting domain prevents complete translocation into the matrix. The matrix targeting domain is removed by MPP/PEP and the mature region of the precursor continues to cross the outer membrane into the intermembrane space. The remainder of the presequence is then removed by inner membrane protease 1.

Panel B

Conservative sorting mechanism. The precursor is imported completely into the matrix and is re-exported to the intermembrane space using a bacterial-like export machinery, after interaction with hsp60.


A
OM
MPP
IM
hsp60
IMP1


B
OM
MPP
IM
hsp60
IMP1

surface receptor (Li and Shore, 1992). Import of the chimaeric protein did not compete with import of matrix proteins, and may thus use a different receptor. The hydrophobic N-terminal region of the outer membrane protein was proposed to act as a signal anchor sequence, preventing complete translocation through the outer membrane.

1.4.4 Mitochondrial Presequence Structure and Function

The structure of mitochondrial protein presequences has been investigated using similar methods as for chloroplast protein presequences, using the complementary approaches of secondary structure computer analysis by comparing many different presequences and spectroscopic analysis of presequences in different environments. The former approach was taken by von Heijne (1986) who analysed 23 mitochondrial targeting signals and discovered that most of them had the potential to form an α-helix with a high hydrophobic moment in a suitable environment. Interestingly, the N-terminus of OMP70 and cytochrome *c* showed no tendency to form an amphiphilic α-helix, perhaps reflecting their different import pathways (section 1.4.3). Amino acid analysis revealed mitochondrial presequences to be rich in alanine, leucine, arginine and serine residues, but deficient in aspartate, glutamate, isoleucine and lysine residues. Intermembrane space proteins have a bipartite presequence consisting of a matrix targeting signal followed by a signal peptide-type sequence (von Heijne *et al.*, 1989).

Roise *et al.* (1986) synthesised a peptide corresponding to the presequence of yeast cox IV which inserted spontaneously into phospholipid monolayers. CD spectrum analysis indicated that this peptide had little secondary structure in an aqueous environment but was partially α-helical in the presence of detergent micelles. Sequence analysis predicted that the peptide was surface active and able to form an amphiphilic α-helix. The disruption of phospholipid vesicles was enhanced by a membrane potential (negative inside) which suggests a role for the pmf across the mitochondrial inner membrane. The peptide was later shown to be imported into

phospholipid vesicles, and thus resistant to added proteases, in a $\Delta\Psi$-dependent manner (Maduke and Roise, 1993), and the possibility was raised that the membrane potential could impart specificity of import into mitochondria rather than other organelles. The peptide could also partition directly into unenergised yeast mitochondria due to electrostatic interactions between the positively charged presequence and negatively charged membrane lipids (Swanson and Roise, 1992), which may be important in the initial stages of precursor binding to mitochondria. Work by Endo and Schatz (1988) confirmed the idea that presequences may fold into a helix only in the presence of membranes. A chimaeric protein consisting of the presequence of cox IV linked to DHFR was synthesised and the DHFR portion shown to fold as for authentic DHFR. This protein had only a weak ability to disrupt vesicles with a low content of negatively charged lipids when compared with the presequence alone. An increase in membrane-disruption could be obtained by either denaturing the protein with urea or increasing the proportion of phospholipids in the vesicles. The increase in phospholipids caused a partial unfolding of the precursor, suggesting that a conformational change may be required for the interaction of this precursor with the mitochondrial membrane. An unfolded structure in an aqueous environment was also shown for a peptide corresponding to the $F_1\beta$ presequence (Bruch and Hoyt, 1992), with α-helix formation induced by the presence of detergent micelles or the solvent trifluoroethanol. Using CD and NMR spectroscopy, a stable helix was shown to form initially at the N-terminus of the peptide followed by another less stable helix at the C-terminus. The initiation of helix formation at two independent regions of the peptide could explain why the $F_1\beta$ presequence contains redundant targeting information.

1.4.5 Comparison of Chloroplast and Mitochondrial Targeting Signals

In the majority of cases, analysis of the presequence of a precursor protein can indicate whether it is targeted to the mitochondrion or chloroplast. The amino acid preferences of the presequences are somewhat different, with the only common feature being that both chloroplast and mitochondrial presequences lack acidic residues. Mitochondrial targeting signals are enriched in arginine, alanine and serine but chloroplast targeting signals have a much higher proportion of serine residues and are also enriched in threonine residues. Mitochondrial presequences can be divided into two domains, an N-terminal domain with a tendency to form an amphiphilic α-helix and a C-terminal domain which tends to have arginine residues at the -2 and/or -3 positions. Stromal presequences tend to have an uncharged N-terminal region deficient in proline and glycine, a central serine-rich domain containing few acidic amino acids and a C-terminal region with a high arginine content and the potential to form an amphiphilic β-sheet. Exceptions to these general features are the presequences of chloroplast precursors from *Chlamydomonas reinhardtii*; for example, the *C. reinhardtii* copper(II)-repressible cytochrome *c* is a chloroplast protein with a presequence which resembles mitochondrial targeting signals (see section 1.3.7).

1.4.6 Proteolytic Processing of Imported Mitochondrial Proteins

Mitochondria contain several peptidases involved in the maturation of mitochondrial precursor proteins. After import into the matrix, the matrix targeting domain of the presequence is removed by the matrix-located mitochondrial processing peptidase, producing either the mature protein or an intermediate. Proteins destined for the intermembrane space usually contain an additional targeting signal which is removed by a protease of the inner membrane. A subset of matrix proteins are cleaved by the general matrix peptidase to an intermediate size and then to the mature size by another matrix peptidase which acts specifically to remove an octapeptide from the intermediate to produce the mature protein.

(a) Mitochondrial Processing Peptidase

A soluble processing peptidase involved in the maturation of mitochondrial precursor proteins was extracted from yeast mitochondria as a complex of MW = 115,000 which dissociated into several subunits upon SDS-polyacrylamide gel electrophoresis. The complex had a pH optimum of 7 - 8 and was a metalloprotein able to cleave matrix proteins to the mature size (McAda and Douglas, 1982) and intermembrane space proteins to an intermediate size (Böhni *et al.*, 1983). Two yeast mutants were isolated by Yaffe and Schatz (1984), *mas1* and *mas2*, which accumulated the precursor to $F_1\beta$ and were later identified as mutants in the two subunits of the mitochondrial processing peptidase. The *MAS1* and *MAS2* genes were cloned (Witte *et al.*, 1988; Jensen and Yaffe, 1988) and shown to encode two structurally related subunits (Pollock *et al.*, 1988) named the processing enhancing protein (PEP, MW 51,000; Hawlitschek *et al.*, 1988) and mitochondrial processing peptidase (MPP, MW 53,000) respectively. Initially, MPP was thought to have a low activity on its own, with PEP increasing this activity, hence the names of the subunits, but it is now thought that both subunits are absolutely required for activity. The *Neurospora crassa* enzyme was isolated by Hawlitschek *et al.* (1988) who showed that PEP was approximately 15-fold more abundant than MPP and was partly associated with the inner membrane whereas MPP was soluble and contained the catalytic site (Yang *et al.*, 1991).

The structure of MPP from yeast and *N. crassa* was investigated by Schneider *et al.* (1990) after cloning of the *N. crassa* enzyme. The two proteins were found to be homologous and to consist of two domains of approximately equal size separated by a loosely structured loop. Four cysteine residues are conserved between yeast and *N. crassa* MPP and inactivation of the proteins by the cysteine-modifying reagent N-ethylmaleimide suggested that at least one of these residues is necessary for activity.

MPP and PEP in some species appear to be bi-functional proteins involved in both precursor processing and electron transport. This was first reported by Schulte *et al.* (1989) who compared the sequence of PEP with those of subunits of the

cytochrome *c* reductase complex. In *N. crassa*, subunit I is identical to PEP and Southern blotting experiments indicated that there is only a single gene encoding the proteins involved in both processing and electron transport. In yeast, subunit I and PEP are related but not identical, and subunit II is also of the same protein family. Subunit I of the bovine cytochrome *c* reductase complex is also homologous to PEP (Gencic *et al.*, 1991), suggesting that the protein family may include members from a diverse range of species. The processing enzymes from both spinach (Eriksson and Glaser, 1992) and potato (Braun *et al.*, 1992) were shown to be membrane bound, localised to the inner membrane in the case of potato, which implies that the import and/or processing mechanisms may be somewhat different in higher plants than in yeast and *N. crassa*. The potato MPP/PEP complex co-purified with the cytochrome *c* reductase complex, and the complex itself was shown to be bi-functional, capable of both precursor processing and participation in electron transport (Braun *et al.*, 1992). Subunit II is similar to *N. crassa* PEP and subunit III to MPP, and these plant subunits may thus be members of this same protein family.

The site of cleavage by MPP/PEP within mitochondrial precursor proteins does not appear to have a highly conserved primary structure, as was seen for SPP. Analysis of the residues around various cleavage sites showed arginine to be enriched at positions -2 and -3, in particular at the -2 position. Secondary structure predictions led to the suggestion that a transition from an α-helix to a random coil-type structure may be involved in defining a cleavage site (von Heijne *et al.*, 1989). The importance of the conserved arginine residue was confirmed by site-directed mutagenesis of a cytochrome b_2-DHFR fusion protein resulting in the arginine at the -2 position being replaced by a glycine residue (Arretz *et al.*, 1991). The mutant could no longer be cleaved by MPP/PEP, showing that the enzyme requires the arginine residue for activity in this case. Mammalian precursor proteins have a lower frequency of arginine residues at this position, which could indicate that the enzyme has a slightly different specificity in different species. A more detailed comparison of a large number of precursor proteins led to the proposal of loosely conserved cleavage motifs

(Gavel and von Heijne, 1990). Those precursors with an arginine residue at position -2 tended to have a serine residue at position +2, giving the motif Arg-X ↓ X-Ser, where X is any amino acid and the arrow indicates the position of cleavage. Precursors with arginine at position -3 tended to contain the motif Arg-X-Tyr ↓ (Ala/Ser), although by comparison with the -2 motif the suggestion was made that the actual cleavage site could be Arg-X ↓ Tyr-(Ala/Ser) with the tyrosine residue removed after cleavage had occurred. There are many exceptions to these motifs, and recognition and cleavage of precursor proteins by MPP/PEP must involve more, as yet unidentified, structural features.

(b) Mitochondrial Intermediate Peptidase

Several matrix or inner membrane proteins are cleaved in two steps, with both reactions catalysed by matrix-located peptidases. MPP/PEP is responsible for the first step, producing an intermediate-sized protein containing an eight residue extension when compared with the mature protein. A matrix enzyme (mitochondrial intermediate peptidase, MIP) in rat liver mitochondria was identified which could remove the octapeptide from these intermediates but which was inactive on precursor proteins (Kalousek *et al.*, 1988). The enzyme was purified as a 75 kDa monomer with a broad pH optimum of between 6.6 and 8.8 which could be inactivated by NEM and EDTA and stimulated by divalent metal cations (Kalousek *et al.*, 1992). Protein sequence comparisons led to the identification of a putative yeast MIP homologue and MIP also had some homology with a subfamily of zinc metalloendoproteases involved in the processing of peptides, particularly in a region containing a potential zinc-binding domain (Isaya *et al.*, 1992).

The function of MIP was investigated by Isaya *et al.* (1991) using the precursor to human ornithine transcarbamylase (pre-OTC), which is normally cleaved by MPP/PEP followed by MIP. Deletion of the octapeptide normally removed by MIP resulted in the inability of both MPP/PEP and MIP to cleave the precursor. When the presequence of the yeast $F_1\beta$ precursor (cleaved by MPP/PEP only) was

linked to the mature part of *N. crassa* ubiquinol-cytochrome *c* reductase iron-sulphur subunit (Fe/S; cleaved by MPP/PEP and MIP), no cleavage was seen by either MPP/PEP or MIP. Together these results suggest that some proteins may have a mature N-terminal which is incompatible with cleavage by MPP/PEP and require a two step cleavage involving MIP to overcome this problem (Isaya *et al.*, 1991; Gavel and von Heijne, 1990).

(c) Inner Membrane Protease I

Some mitochondrial precursor proteins destined for the intermembrane space and inner membrane are synthesised with a bipartite presequence, the N-terminal domain of which is removed by MPP/PEP and the C-terminal domain then directs the correct localisation of the protein. A yeast mutant was identified which accumulated the nuclear-encoded protein cytochrome b_2 as an intermediate form and the mitochondrially-encoded protein cytochrome oxidase subunit II (cox II) as the precursor form. Complementation of this mutant allowed cloning of a gene encoding inner membrane protease I (IMP 1; Behrens *et al.*, 1991), a protease capable of processing cytochrome b_2 and cox II to the mature size. The mutant contained correctly processed cytochrome c_1 and cytochrome *c* peroxidase, suggesting that these proteins are cleaved by a different protease. IMP 1 is an integral membrane protein exposed on the outer face of the inner membrane which requires divalent cations and acidic phospholipids for activity. The enzyme contains a 21.4 kDa subunit, antibodies against which inhibit IMP 1 activity, but overproduction of this subunit does not increase activity and the enzyme may therefore be a hetero-oligomer (Schneider *et al.*, 1991). IMP 1 contains sequence homology to both *E. coli* leader peptidase and the E.R. signal peptidase, and is thus a member of the signal peptidase family including these two enzymes and also the chloroplast TPP (Behrens *et al.*, 1991).

1.5 Export of Proteins Across the Cytoplasmic Membrane of Escherichia coli

E. coli periplasmic proteins are synthesised in the cytoplasm and exported across the cytoplasmic membrane by a mechanism which often depends on the presence of an N-terminal extension or leader peptide.

1.5.1 Translocation Across the Cytoplasmic Membrane

The export of most *E. coli* proteins into the periplasm is dependent on the *sec* gene products which constitute the translocation machinery of the cytoplasmic membrane, and requires the hydrolysis of ATP and a pmf across the membrane (Driessen, 1992). Proteins are thought to be translocated in an unfolded conformation, and unfolding can be driven by the translocation event itself (Arkowitz *et al.*, 1993). The *secA* gene product is a translocation ATPase which passes through a cycle of membrane binding and dissociation during protein export. A complex of SecA, ATP and the precursor protein binds to the membrane, hydrolysis of the ATP causes release of the precursor protein from SecA and partial translocation into the membrane, and the completion of translocation is independent of the presence of SecA (Johnson *et al.*, 1992). SecA thus has a dual function, being involved in targeting to the membrane and in the initiation of translocation (Swiderska *et al.*, 1990). Some proteins (*eg* maltose binding protein, MBP) require cytoplasmic chaperones such as SecB, trigger factor or GroEL for export to maintain export competence (Lecker *et al.*, 1989). SecD is involved in the release of proteins from the cytoplasmic membrane after translocation is completed (Matsuyama *et al.*, 1993) whereas SecY has been proposed to be a component of a proteinaceous channel through which precursor proteins may pass (Joly and Wickner, 1993). Other *sec* gene products have other, as yet undefined, roles in protein export. An *E. coli* preprotein translocase has been isolated which is capable of catalytically transporting proteins across a membrane *in vitro* (Bassilana and Wickner, 1993) and shown to consist of SecA bound to the integral membrane proteins SecY/E. A subset of proteins, however, can be translocated across the membrane even when Sec function is

completely blocked, implicating the presence of a Sec-independent pathway of export, and a linear correlation has been shown between the length of the translocated section and the dependence of the protein on the *sec* gene products for translocation (Andersson and von Heijne, 1993). The *E. coli* protein pre-β-lactamase required SRP for translocation across the mammalian ER membrane, indicating that *E. coli* proteins can interact functionally with SRP (Müller *et al.*, 1982) and therefore that an *E. coli* homologue is likely to exist. An *E. coli* complex with homology to the mammalian signal recognition particle (SRP) has been identified and shown to interact specifically with leader peptides (Luirink *et al.*, 1992; Hartl and Wiedmann, 1993) but it is not yet clear whether it acts specifically in the targeting of precursor proteins to the cytoplasmic membrane or simply as a chaperone to maintain translocation competence.

1.5.2 Leader Peptide Structure and Function

Leader peptides have a structure similar to that of thylakoid targeting signals, consisting of three domains; a positively charged N-terminus, a hydrophobic core region and a leader peptidase (LEP) cleavage site (Johnson *et al.*, 1992). The importance of the hydrophobic region for membrane translocation was demonstrated by the sequencing of the leader peptides of MBP mutants whose export was inhibited. In the majority of mutants, export failure was due to the introduction of a single charged residue into the hydrophobic core region (Bedouelle *et al.*, 1980). A series of mutants of *E. coli* alkaline phosphatase were created by Doud *et al.* (1993) with hydrophobic core regions consisting of different ratios of alanine to leucine residues. The translocation-competence of these mutants demonstrated that leader peptide function shows a non-linear dependence on the hydrophobicity of the core region. The conformation of the leader peptide of the λ phage receptor in different environments was investigated by Briggs *et al.* (1986) who found that in aqueous environments the leader peptide was relatively unstructured, whereas binding to a lipid monolayer induced formation of a β-structure and insertion into the monolayer

caused an α-helical structure to form. This α-helix formation was shown by CD spectral analysis of the PhoE leader peptide to require acidic phospholipids (Keller *et al.*, 1992) and interaction of this leader peptide with the membrane destabilised the lipid bilayer, promoting the formation of non-bilayer structures (Killian *et al.*, 1990). However, a leader peptide was found to cause the opening of a large aqueous trans-membrane channel *in vitro*, with the leader peptide binding within the mouth of the pore, providing further evidence that the protein is translocated through a proteinaceous channel rather than directly through the lipid bilayer (Simon and Blobel, 1992). Leader peptides are now thought to function by adopting an α-helical conformation during spontaneous insertion into the cytoplasmic membrane and then diffusing in the plane of the membrane until they come into contact with the Sec protein translocation machinery (Johnson *et al.*, 1992). The role of an SRP-type protein in this scheme is unclear.

1.5.3 Leader Peptidase

After translocation across the cytoplasmic membrane, the leader peptide is removed from the *E. coli* precursor proteins by leader peptidase (LEP), an enzyme of the signal peptidase family (Dalbey and von Heijne, 1992; Black *et al.*, 1992). The *lep* gene encodes a 36 kDa integral membrane protein with a large domain exposed on the outer surface of the cytoplasmic membrane (Wolfe *et al.*, 1983). Site-directed mutagenesis studies and sequence comparisons of various *E. coli* precursor proteins have identified features of the leader peptides which are important in recognition by LEP. Small, neutral amino acids at the -1 and -3 positions with respect to the LEP cleavage site were shown to be essential for cleavage by LEP, preceded by a helix-disrupting residue such as proline or glycine (Kuhn and Wickner, 1985; Dierstein and Wickner, 1986; Shen *et al.*, 1991). Blockage of processing at the authentic site led, in the case of MBP, to cleavage at another site which also contained the features required for cleavage (Fikes *et al.*, 1990), as has also been seen upon mutagenesis of the TPP cleavage site in a thylakoid lumen precursor protein (Shackleton and

Robinson, 1991). An MBP mutant containing a proline residue at the +1 position was also unable to be cleaved by LEP, although binding to the enzyme still occurred (Barkocy-Gallagher and Bassford, 1992), implying that the mature protein also contains features required for recognition by LEP.

E. coli glyceride-modified prolipoproteins are not processed by LEP but by another processing peptidase of the cytoplasmic membrane, prolipoprotein signal peptidase (LSP). Presumably, LEP is unable to recognise these modified proteins and so a specialised enzyme is required (Muñoa *et al.*, 1991).

1.6 Protein Secretion in Eukaryotic Cells

Proteins destined for secretion in eukaryotic cells are synthesised on ribosomes bound to the endoplasmic reticulum (ER) membrane and translocated co-translationally into the ER lumen, from which they are transported to the cell surface. This process has been studied in some detail and the mechanisms by which proteins are targeted to the ER membrane are well understood in comparison with targeting in many other systems.

1.6.1 Translocation of Proteins Across the Endoplasmic Reticulum Membrane

The targeting of proteins to the ER is a signal sequence-mediated process involving protein components in the cytosol, ER membrane and ER lumen. The synthesis of secreted proteins begins on free ribosomes but once the signal sequence emerges from the ribosome, the signal recognition particle (SRP) binds to the signal sequence via a methionine-rich domain of the 54 kDa subunit (Lütcke *et al.*, 1992) and to the ribosome, slowing down the elongation process and mediating the binding of the ribosome-nascent chain-SRP complex to the signal recognition particle receptor (SR) which is essential for translocation and contains a functional GTP-binding site (Rapiejko and Gilmore, 1992). The signal sequence binds to a non-essential signal sequence receptor (SSR) in the ER membrane (Migliaccio *et al.*, 1992), identified by cross-linking to the signal sequence of a nascent protein

(Wiedmann *et al.*, 1987). A ribosome receptor is also thought to aid binding of the complex to the ER membrane (Kreibich and Sabatini, 1992). The SSR then binds GTP and the signal sequence and ribosome are released from SRP. Hydrolysis of the GTP then allows the release of SRP from the receptor protein (Rapoport, 1990).

The translocation steps through the membrane after targeting has occurred are less well defined. After membrane binding, the secretory protein is thought to be transferred to a second receptor, which in yeast may be the Sec62 and Sec63 proteins. It is then transferred to the "translocon" which is responsible for the translocation of the protein across the membrane and has been proposed to contain the Sec61 protein. ATP is required for translocation, possibly for interaction of the translocating protein with Sec61. Other ER membrane proteins have been shown to be involved in translocation and it has been suggested that translocation involves the sequential interaction of the secretory protein with various proteins of the ER membrane. A molecular chaperone in the ER lumen (Binding Protein or BiP) binds to the protein as it emerges from the membrane to allow correct folding and also binds misfolded proteins which are not competent for transport to the Golgi (Sanders and Schekman, 1992). BiP requires ATP for activity and this is provided by an ATP transporter in the ER membrane, inhibition of which blocks protein translocation (Mayinger and Meyer, 1993). The signal sequence is then removed by signal peptidase. From the ER lumen, the protein is transported via coated vesicles to the Golgi network and then to the cell surface or other compartments such as the lysosome or vacuole, with post-translational modifications such as asparagine-linked glycosylation occurring during this pathway (Rothman and Orci, 1992).

1.6.2 Signal Peptide Structure and Function

Eukaryotic signal peptides are highly similar to prokaryotic leader peptides in both structure and properties, consisting of three domains as described in section 1.5.2. Slight differences in overall properties have been described by von Heijne (1985); eukaryotic signal peptides can be slightly shorter than leader peptides and the

n-region in eukaryotes tends to have one more positive charge than in prokaryotes. However, the structural features of signal sequences and leader peptides which can be identified appear to be very similar, despite the lack of primary structure homology between sequences.

The function of signal peptides appears to be the same as that of *E. coli* leader peptides, having similar properties, for example, a signal peptide was shown to alter the structure of a lipid bilayer, destabilising the bilayer, and to adopt an α-helical conformation in the presence of membranes (Tahara *et al.*, 1992). Similar properties were also described for leader peptides (section 1.5.2).

1.6.3 Signal Peptidase

The eukaryotic signal peptidase (SP) is an ER membrane protein with its active site on the lumenal face of the membrane. It is a member of the peptidase family also containing LEP, TPP and IMP 1 (Dalbey and von Heijne, 1992) and has a reaction specificity similar to these other peptidases.

1.7 Protein Import into Peroxisomes

Peroxisomes are small organelles bounded by a single membrane which contain catalase and some enzymes of the β-oxidation pathway. Peroxisomal matrix and membrane proteins are synthesised in the cytosol and imported post-translationally into the organelle, usually without a proteolytic processing step, and the information for targeting to the peroxisome must therefore reside in the mature protein.

1.7.1 Peroxisomal Protein Translocation Machinery

Little is yet known about the mechanism by which proteins cross the peroxisomal membrane. Analogy with the chloroplast and mitochondrial systems suggests that import is likely to occur via proteinaceous receptors in the peroxisomal membrane, and the existence of several different classes of targeting signal (see

section 1.7.2) implies that there may be several different import receptors. Analysis of mutant cells which are peroxisome-deficient has led to the identification of several genes which may encode components of the peroxisomal import apparatus, but this remains to be confirmed (Lazarow, 1993). The *in vitro* import of the peroxisomal protein acyl-CoA oxidase requires the presence of ATP but not a membrane potential, but it has yet to be shown whether this is the general situation (de Hoop and Ab, 1992).

1.7.2 Peroxisomal Targeting Signals

Studies using firefly luciferase identified the first known peroxisomal targeting signal, situated at the C-terminus of the protein. This consisted of a tripeptide serine-lysine-leucine and was found to be sufficient to direct reporter proteins into peroxisomes. Sequence analysis and mutagenesis studies allowed the identification of a minimal targeting signal with the consensus sequence (Ser/Cys/Ala)-(Lys/His/Arg)-Leu. This motif is often found in mammalian proteins but is less frequent in lower eukaryotic proteins, implying the existence of other targeting signals. Acyl-CoA oxidase of *Candida tropicalis* contains internal sequences which are capable of targeting a reporter protein to yeast peroxisomes and a few peroxisomal proteins are synthesised with N-terminal presequences which are removed during import (de Hoop and Ab, 1992). Nothing is known about the enzyme catalysing this proteolysis step.

1.8 Summary

Although different mechanisms appear to be involved in the targeting of proteins across the various membranes of the cell, several common features can be identified.

1.8.1 Role of Receptors

Proteinaceous receptors have been implicated in the binding of proteins to the membrane in most protein translocation systems. In chloroplasts, there is evidence that a 30 kDa protein in the envelope membrane may be an import receptor (Pain *et al.*, 1988; Kaderbhai *et al.*, 1988). Two mitochondrial receptor proteins with overlapping specificities have been identified, MOM72 and MOM19 (Segui-Real *et al.*, 1992) and another outer membrane protein of 32 kDa may also be involved (Murakami *et al.*, 1990). A signal sequence receptor in the ER membrane is the site of recognition of the SRP-nascent chain-ribosome complex prior to translocation (Rapoport, 1990) and SecY may play a similar role in the *E. coli* cytoplasmic membrane (Johnson *et al.*, 1992).

1.8.2 Membrane Translocation

The mechanism by which a protein is translocated across a membrane is still largely unknown. It is thought that proteins may be transported through a proteinaceous channel in the membrane rather than being in contact with the membrane lipids themselves, but the proteins which make up this channel have not yet been identified in many cases. Several ER membrane proteins have been implicated in the translocation process (Sanders and Schekman, 1992) and some of the *E. coli* Sec proteins are thought to form a channel in the membrane (Joly and Wickner, 1993). The protein translocation system in the mitochondrial outer membrane has been isolated and its protein constituents identified (Söllner *et al.*, 1992) but the inner membrane system remains to be characterised. Mitochondrial (Wienhues *et al.*, 1991) and chloroplast (Pain *et al.*, 1988; Schnell and Blobel, 1993) proteins are transported across the membrane at sites of contact between the outer and inner membranes and proteins are probably transported across the membrane in an unfolded state (Pilon *et al.*, 1992; Eilers and Schatz, 1986; Wienhues *et al.*, 1991).

1.8.3 Targeting Signals

N-terminal presequences targeting proteins to different membranes fall into two broad classes. The first contains ER targeting signals, *E. coli* export signals, chloroplast TTDs and possibly mitochondrial intermembrane space targeting signals which consist of a hydrophilic N-terminal domain, a central hydrophobic core region and a C-terminal peptidase cleavage site (Verner and Schatz, 1988). The second contains chloroplast stroma and mitochondrial matrix targeting signals which are hydrophilic in nature, are rich in hydroxylated residues and have a net positive charge (von Heijne *et al.*, 1989). The first group of targeting signals appear to share a similar mechanism of targeting as, for example, a TTD can act as an export signal in *E. coli* (Meadows and Robinson, 1991). In contrast, mitochondrial and chloroplast targeting signals are generally very specific (Smeekens *et al.*, 1987) and few examples of mis-targeting are known.

1.8.4 The Role of Chaperones in Protein Targeting

Molecular chaperones appear to be involved in various stages of protein targeting in several systems, both in maintaining precursor proteins in a translocation-competent conformation prior to membrane transport and in the folding and assembly of proteins after translocation is complete to prevent unproductive protein interactions occurring (Eilers and Schatz, 1988; Tsugeki and Nishimura, 1993).

1.8.5 Energy Requirements for Translocation

ATP is required by most of the protein translocation systems which have been characterised and seems to be involved in the initial stages of transport, possibly for the unfolding of precursors to allow translocation (Eilers and Schatz, 1988). A pmf is also required for protein translocation across some membranes; the thylakoid membrane requires the ΔpH component of the pmf (Mould and Robinson, 1991), the mitochondrial envelope system requires the $\Delta\Psi$ component (Eilers *et al.*, 1987) and the *E. coli* cytoplasmic membrane is able to use either component (Geller, 1991). The

chloroplast envelope membrane, ER membrane and probably the peroxisomal membrane do not require a pmf for protein translocation.

1.8.6 Processing Peptidases

The processing peptidases which remove ER signal peptides, *E. coli* leader peptides, chloroplast TTDs, and possibly mitochondrial intermembrane space targeting signals appear to be similar in reaction specificity and mechanism. These peptidases are not typical of any of the standard families of proteases and Dalbey and von Heijne (1992) suggested that they may belong to a new family of serine proteases, related to the β-lactamases. The peptidases are integral membrane proteins with their active site on the *trans* side of the translocating membrane and the peptidases whose structures are known show some similarities to each other. The specificities of the peptidases are also similar. The *E. coli* and ER peptidases have almost identical requirements for small residues at the -1 and -3 positions, whereas TPP has a similar but narrower specificity.

SPP and MPP are not members of this peptidase family, having entirely different substrate specificities and mechanisms to the signal-type peptidases. They are both metalloproteases (Robinson and Ellis, 1984a; McAda and Douglas, 1982), usually soluble although MPP has been reported to be membrane-bound in some cases (Eriksson and Glaser, 1992; Braun *et al.*, 1992). The requirements for recognition by these proteases are still largely unknown and it is thought that elements of secondary or tertiary structure are involved.

1.8.7 The Endosymbiotic Theory of Organelle Evolution

The endosymbiotic theory is based on the resemblance that mitochondria and chloroplasts have to bacterial cells and the similarities between organelle and prokaryotic gene sequences. It proposes that mitochondria and chloroplasts arose from the internalisation of a bacterium (photosynthetic bacterium in the case of chloroplasts) into a primitive ancestral cell and the establishment of a symbiotic

relationship between them. Gene transfer from the bacterium to the host cell nucleus was proposed to then occur, producing the present situation whereby most mitochondrial and chloroplast proteins are nuclear-encoded (Schwartz and Dayhoff, 1978). This is also reflected in protein targeting systems, as thylakoid lumen proteins, for example, contain TTDs which resemble the presequences of lumenal proteins of free-living cyanobacteria (Kuwabara *et al.*, 1987). Targeting signals for translocation across the chloroplast envelope or mitochondrial membranes must therefore have evolved concomitantly with the transfer of genes to the nucleus, and once inside the organelle proteins could undergo 'conservative sorting', using the ancestral prokaryotic targeting pathway to reach their sub-organellar location.

1.9 Aims of Project

The processing peptidases involved in the maturation of precursor proteins are of great interest, as generally the precursor forms of translocated proteins are inactive and the peptidases involved in their maturation are therefore essential to cellular function. These peptidases are also known to be highly specific, yet very little is known about the basis for this specificity. The stromal processing peptidase processes many essential proteins upon their import into the chloroplast, but the features recognised by this enzyme are elusive. This project had the following aims:-

(1) To produce a highly purified preparation of SPP.

(2) To determine the site of SPP cleavage within the presequences of some thylakoid lumen proteins.

(3) To investigate the requirements for the cleavage of a thylakoid lumen protein by site-directed mutagenesis.

(4) To compare the reaction specificities of SPP and MPP.

CHAPTER 2 - MATERIALS AND METHODS

2.1 *Bacterial Strains*

Strain	Reference
TG1	Sambrook *et al.*, (1989)
JM109	Sambrook *et al.*, (1989)

2.2 *Growth and Maintenance of* Escherichia coli *strains*

E. coli stock strains were plated on L-agar medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 170 mM NaCl, 1.5% (w/v) agar) and stored at 4°C. Liquid cultures of *E.coli* were grown in L-broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 170 mM NaCl) and maintained at 37°C in an orbital shaker at 200 rpm. Cells containing plasmids conferring ampicillin resistance were grown in the presence of 50 µg/ml ampicillin.

2.3 *Preparation of Competent Cells*

E. coli cells were grown to an OD_{600} of 0.5 in a 10 ml culture of L-broth with shaking. Cells were then centrifuged for 5 minutes at 2500 *g*, the supernatant removed, and the pellet of cells resuspended in 5 ml of 100 mM $CaCl_2$ at 0°C. The suspension was placed on ice for 20 minutes and the cells pelleted by centrifugation as before. The pellet was resuspended in 1 ml of fresh 100 mM $CaCl_2$ at 0°C and stored for up to 24 hrs on ice before use.

2.4 *Transformation of Competent Cells*

Control transformations were always carried out as follows:

(a) transformation with no DNA added to check for contamination.

(b) transformation containing 1 ng of double-stranded DNA to determine the efficiency of transformation.

2.4.1 Plasmid DNA

Approximately 1 ng of DNA was added to 100 µl of competent cells and the cells placed on ice for 45 to 120 minutes with intermittent mixing. The cells were heat-shocked at 42°C for 2 minutes and diluted to 1 ml with L-broth. After incubation at 37°C for 30 minutes the cells were pelleted by centrifugation in a microcentrifuge for 1 minute and resuspended in 100 µl of L-broth. Cells were plated on L-agar plates containing ampicillin if required and incubated overnight at 37°C.

2.4.2 M13 DNA

Transformations were carried out essentially as for plasmid DNA except that 200 µl of competent cells were used and after heat-shock the cells were placed on ice for 5 minutes and then added to 3 ml of H-top (1% (w/v) tryptone, 135 mM NaCl, 0.8% agar) containing 200 µl of an overnight culture of *E. coli* and 40 µl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-10-β-D-galactoside (x-gal) and 20 mg/ml isopropylthio-β-D-galactoside (IPTG) if required. This mixture was poured onto H-plates (1% (w/v) tryptone, 135 mM NaCl, 1.5% agar) and incubated overnight at 37°C.

2.5 Preparation of Phenol

100 g of phenol was dissolved in 100 ml of 1.5 M Tris (tris(hydroxymethyl)methylamine)-Cl pH 8.0 and 8-hydroxyquinoline (0.1% (w/v)) added to colour the phenol and to act as an antioxidant. The top (aqueous) phase was removed and fresh 1.5 M Tris-Cl pH 8.0 mixed with the organic phase. This process was repeated until a pH of between 7.5 and 8.0 was reached. The aqueous layer was then replaced with 10 mM Tris-Cl pH 8.0 and the phenol stored in aliquots at -20°C.

2.6 Phenol/Chloroform Extraction of DNA

DNA in aqueous solution was mixed with an equal volume of phenol/chloroform (1:1) and left at room temperature for 15 minutes with intermittent mixing. The phenol and aqueous layers were separated by centrifugation in a

microcentrifuge for 3 minutes. The upper aqueous layer containing the DNA was removed, placed into a fresh tube and mixed with an equal volume of chloroform. The layers were again separated by centrifugation and the aqueous layer removed to a fresh tube.

2.7 Precipitation of DNA

The DNA-containing solution was mixed with 0.1 volumes of 3 M sodium acetate pH 5.6 and 2 volumes of ethanol or 0.6 volumes of isopropanol, depending on the volume of the starting solution. The mixture was placed in an ethanol/dry ice bath until frozen to precipitate the DNA, which was then pelleted by centrifugation at high speed in a microcentrifuge for 20 minutes. The ethanol was removed and the pellet dried under vacuum and resuspended in water.

2.8 Preparation and Running of an Agarose Gel

0.4 g of agarose was mixed with 50 ml of 1xTBE (90 mM Tris-borate, 2 mM ethylenediaminetetraacetic acid (EDTA)) and heated in a microwave oven until the agarose was completely dissolved to produce a 0.8% (w/v) solution of agarose, and ethidium bromide added to a final concentration of 0.5 µg/ml after the solution had cooled to below 60°C. The gel mixture was cast in a BRL "Horizon 58" horizontal slab gel former and a slot-former inserted. When the gel had set the slot-former was removed and the DNA samples loaded after being mixed with 0.2 volumes of DNA sample buffer (50% (v/v) glycerol in water and 0.25% bromophenol blue). The gel was run in 1xTBE according to the manufacturers instructions and DNA bands visualised using a u.v. transilluminator.

2.9 Extraction of DNA Fragments from an Agarose Gel

Based on Girvitz *et al.* (1980).

DNA was digested with restriction enzymes as required and the fragments produced separated on a 0.8% (w/v) agarose gel. The required band was identified by

illumination with u.v. light and a slit cut in the gel just ahead of the band (*ie* on the anode side). A piece of Whatman no.1 filter paper pre-soaked in sterile 1xTBE was placed into the slot and a piece of dialysis membrane prepared by boiling in 1 mM EDTA and rinsed in sterile distilled water was placed in front of the paper to prevent the DNA from passing through the paper. The gel was run for a further 30 minutes to allow the DNA fragment to run into the Whatman paper and the current reversed for 10 seconds to ensure that any DNA adhering to the dialysis membrane was removed. The paper containing the DNA was then placed in an Eppendorf tube and mixed with 200 µl of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). A hole was punctured in the bottom of the tube, the tube placed inside another, and the liquid centrifuged through into the lower tube. The paper was washed with further aliquots of TE buffer until all of the DNA was extracted. The dissolved DNA was phenol/chloroform extracted, ethanol precipitated, dried and redissolved in water.

2.10 Digestion of DNA using Restriction Endonucleases

Restriction enzymes were used with their recommended buffers according to the manufacturer's protocol. 1 to 5 units of restriction endonuclease was added per µg of DNA to be digested.

2.11 Treatment of Linearised Plasmid DNA with Alkaline Phosphatase

Calf intestinal alkaline phosphatase removes 5'-terminal phosphate groups from linearised plasmid DNA and so prevents recircularisation of the vector during a ligation reaction. Vector DNA from a restriction digest was incubated with 1 unit of calf intestinal phosphatase (CIP) for 30 minutes at 37°C. A further unit of CIP was added and the incubation repeated. The enzyme was inactivated by heating to 70°C for 15 minutes and the DNA phenol/chloroform extracted, ethanol precipitated, dried and resuspended in water.

2.12 Ligation of DNA Fragments

Vector DNA (20 ng) was mixed with isolated fragment DNA (100 ng) in ligase buffer (50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 10 mM adenosine triphosphate (ATP)) and incubated overnight at 15°C with 0.1 units/µl of T4 DNA ligase. Competent *E.coli* cells were then transformed with the ligation mixture.

2.13 Mini Preparation of Plasmid DNA

Holmes and Quigley (1981).

Single colonies from L-amp plates of transformed *E. coli* were used to inoculate 10 ml aliquots of L-broth containing 50 µg/ml ampicillin and cultures grown overnight at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation at 2500 *g* for 5 minutes and resuspended in 100 µl of 25% (w/v) sucrose, 50 mM Tris-Cl pH 8.0. The suspension was transferred to an Eppendorf tube and 600 µl of M.STET (5% (w/v) sucrose, 50 mM Tris-Cl pH 8.0, 50 mM EDTA, 5% (v/v) Triton X-100) and 7 µl of lysozyme (50 mg/ml freshly prepared in 50 mM Tris-Cl pH 8.0) were added. The mixture was boiled immediately for 1 minute in a boiling water bath and centrifuged for 45 minutes in a microfuge. The supernatant was removed to a fresh tube, 1 µl of 10 mg/ml ribonuclease A (RNase A) added, and incubated at 37°C for 30 minutes. The sample was phenol/chloroform extracted and the DNA precipitated with isopropanol. The final DNA pellet was resuspended in 50 µl of water.

2.14 Large Scale Preparation of Plasmid DNA

Based on Promega Biotec Technical Bulletin No.9.

10 ml of L-Broth containing ampicillin at 50 µg/ml was inoculated with the plasmid-carrying strain of *E. coli* and the culture grown for 8 hrs with agitation at 37°C. 2.5 ml of this culture was used to inoculate 250 ml of L-Broth containing ampicillin (50 µg/ml) and the large culture grown overnight at 37°C with agitation.

The cells were harvested by centrifugation at 2000 *g* for 10 minutes at 4°C, resuspended in 4 ml of TES (25 mM Tris-Cl pH 8.0, 10 mM EDTA, 15% (w/v) sucrose, 2 mg/ml lysozyme) and left to stand on ice for 15 minutes. 8 ml of 200 mM NaOH, 1% (w/v) sodium dodecyl sulphate (SDS) was added, the sample mixed gently until transparent and placed on ice for 10 minutes. 5 ml of 3 M sodium acetate (pH 4.6) was then added, mixed gently until a dense white precipitate appeared and the mixture placed on ice for 15 minutes. The precipitate was removed by centrifugation at 4°C for 15 minutes at 15000 *g* and the clear supernatant removed. After addition of 5 µl of 10 mg/ml RNase A, the supernatant was incubated at 37°C for 30 minutes. The sample was then phenol/chloroform extracted, isopropanol precipitated and the precipitate resuspended in 400 mM NaCl, 6.5% (w/v) polyethylene glycol (PEG)-6000. The mixture was placed on ice for 60 minutes, the DNA precipitate pelleted by centrifugation for 10 minutes in a microfuge and the supernatant removed. The pellet was washed with 200 µl of 70% (v/v) ethanol and the dried pellet resuspended in 50 µl of water.

2.15 DNA Sequencing using M13 Vector

Based on the method of Sanger *et al.* (1977). Reagents were obtained from United States Biochemical Corporation.

This method requires a single-stranded DNA template for sequencing and the bacteriophage M13 was exploited to generate this template by subcloning the DNA to be sequenced into the single-stranded bacteriophage vector M13mp19. After infection of bacteria, the cells release bacteriophage particles into the growth medium which contain single-stranded DNA, which can then be purified for sequencing.

2.15.1 Growth and Maintenance of M13mp19 Bacteriophage

Vectors were stored as DNA (not bacteriophage) and phage particles regenerated by transformation of competent TG1 cells which were then plated onto H-plates in 3 ml of melted H-top containing 200 µl of an overnight culture of TG1

cells, 40 µl of IPTG and 40 µl of x-gal. Plates were incubated overnight at 37°C and plaques picked using a drawn out Pasteur pipette. M13mp19 contains the coding information for the first 146 amino acids of the β-galactosidase gene (*lac z*) which will complement host cells containing a defective β-galactosidase gene. The plaques formed on plating out these transformed cells are therefore blue in the presence of IPTG and x-gal. Vectors containing a foreign gene inserted into the polylinker region can no longer complement the defective gene and so produce colourless plaques.

2.15.2 Preparation of Single-Stranded Template for Sequencing

1.5 ml of TY (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 85 mM NaCl) was inoculated with 15 µl of an overnight culture of TG1 cells and a single plaque of bacteriophage M13mp19 containing the DNA to be sequenced. This was incubated at 37°C at 250 rpm for 5 hrs. The culture was transferred to an Eppendorf tube and the cells pelleted by centrifugation for 5 minutes in a microcentrifuge. The supernatant was transferred to a fresh tube and centrifuged as before to ensure the removal of all cells. This supernatant was added to 200 µl of 20% PEG-6000 in 2.5 M NaCl and left to stand at room temperature for 15 minutes. The mixture was centrifuged for 5 minutes and the supernatant discarded. The tube was recentrifuged for 2 minutes and the remaining supernatant removed with a drawn out Pasteur pipette. The viral pellet was resuspended in 100 µl of TE buffer and phenol/chloroform extracted. The DNA was ethanol precipitated and the dried pellet resuspended in 30 µl of water.

2.15.3 Sequencing of Single-Stranded DNA

1-2 µg of single-stranded DNA was annealed with 0.5 pmol of universal primer in a total volume of 10 µl of annealing buffer (40 mM Tris-Cl pH 7.0, 20 mM $MgCl_2$, 50 mM NaCl) by heating to 65°C for 2 minutes and allowing to cool slowly to below 35°C over 30 minutes. To the annealed template-primer was added 1 µl of 100 mM DTT, 2 µl of labelling mix (1.5 µM dCTP, 1.5 µM dGTP, 1.5 µM dTTP), 0.5 µl of [α-^{35}S]dATP (10 µCi/µl) and 2 µl of Sequenase enzyme (1.6 units/µl) and

the mix incubated at room temperature for 5-10 minutes. After the labelling reaction, 3.5 µl of labelled template-primer was added to tubes containing 2.5 µl of ddATP (80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddATP, 50 mM NaCl), ddCTP (80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddCTP, 50 mM NaCl), ddGTP (80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddGTP, 50 mM NaCl), ddTTP (80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddTTP, 50 mM NaCl) termination mixes respectively, prewarmed to 37°C. These were then incubated at 37°C for 5 minutes and the reaction stopped by the addition of 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% xylene cyanole FF). The samples were heated for 2 minutes at 70°C before loading onto a sequencing gel.

2.15.4 Preparation and Electrophoresis of a Sequencing Gel

Gel plates and spacers were assembled to provide a mould for a 20x40x0.04 cm slab gel. A 6% acrylamide/0.3% bisacrylamide/700 mM urea in 0.5xTBE gel solution was prepared, 240 µl of 10% (w/v) ammonium persulphate, 40 µl of N,N,N',N'-tetramethylethylenediamine (TEMED) added to 40 ml of this solution and the solution poured into the mould. A slot was formed in the gel to accept a comb and the gel allowed to polymerise for 2 hrs. Once polymerised, the slot was rinsed to remove any urea or unpolymerised acrylamide, the gel assembled in the gel tank (Scieplas, U.K.) and a comb inserted. The gel was pre-run with 0.5xTBE in the upper reservoir, 1xTBE in the lower reservoir at a constant voltage of 1500 volts for 20 minutes to warm the gel and maintain a denaturing environment. Samples from the sequencing reaction were loaded and electrophoresed at a constant voltage of 1500 volts for 3 hrs.

2.15.5 Fixing of a Sequencing Gel

After completion of electrophoresis, the gel was removed from the gel tank and the upper gel plate removed. With the gel remaining on the lower plate for support, it was placed into sequencing gel fixing solution (10% (v/v) methanol, 10% (v/v) acetic acid) for 20 minutes.

2.15.6 Drying and Autoradiography

The fixed gel was placed onto 3 MM Whatman filter paper and placed on a vacuum dryer (Atto, Japan). Cling film was placed over the gel and vacuum and heat applied for 1 hr. The dried gel was removed, placed into a lightproof cassette and exposed to a piece of X-ray film. The film was developed under darkroom conditions as described for SDS-PAGE (section 2.21.6).

2.16 Plasmid Sequencing

DNA sequencing was also performed directly in a recombinant pGEM-4Z plasmid using a method based on that for M13 sequencing. To generate the single stranded DNA required for sequencing, the plasmid was denatured using NaOH. 2 µl of 2 M NaOH was added to 1 µg of DNA in 8 µl of water and left to stand at room temperature for 10 minutes. The DNA was ethanol precipitated and the dried pellet resuspended in 7 µl of water. The sequencing reactions were then carried out as for M13 sequencing using primers complementary to the SP6 or T7 promoter regions of the pGEM vector.

2.17 Oligonucleotide-Directed In Vitro *Mutagenesis*

Site-specific mutagenesis was carried out using an Amersham kit according to the manufacturers instructions. A mutagenesis efficiency of approximately 80% (mutant over non-mutant plaques) was observed. This high efficiency was due to inclusion of a strand-specific selection step made possible by incorporation of a thionucleotide into the mutant strand during synthesis, which renders it resistant to

digestion by *Nci*I. The non-mutant strand can therefore be digested using *Nci*I and removed by exonuclease III digestion, thus preventing host-mediated repair of the mutant strand. In addition, a filtration step is included to remove contaminating single-stranded DNA.

2.17.1 Synthesis of Mutagenic Oligodeoxynucleotides

Oligonucleotides were synthesised using an Applied Biosystems 380B DNA synthesiser and purified using high pressure liquid chromatography (HPLC).

2.17.2 Phosphorylation of Oligonucleotides

The oligonucleotide solution was diluted to 20 nmol/ml in water. To 2.5 µl of this diluted solution was added 3 µl of 10x kinase buffer (1 M Tris-Cl pH 8.0, 100 mM $MgCl_2$, 70 mM DTT, 10 mM ATP), 25 µl of water and 2 units of T4 polynucleotide kinase. This mixture was incubated at 37°C for 15 minutes before heat inactivation of the enzyme by heating to 70°C for 10 minutes.

2.17.3 Preparation of Single-Stranded Template DNA

M13mp19 vector containing the 320 bp *Eco*R1/*Kpn*1 fragment of p33K-2 (Meadows *et al.*, 1991) was provided by Dr J. W. Meadows (Warwick).

To prepare single-stranded DNA, 20 ml of TY was inoculated with 20 µl of a TG1 culture grown overnight and growth was continued at 37°C for 3 hrs. 2 ml of fresh TY was inoculated with 200 µl of the 3 hr culture and a recombinant plaque and incubated for 4 hrs at 37°C and 300 rpm. The cells were centrifuged for 5 minutes in a microfuge and the supernatant containing single-stranded phage was stored overnight at 4°C. 100 ml of TY medium was inoculated with 1 ml of an overnight culture of TG1 and grown at 37°C for 1 hr. The phage supernatant as isolated above was added and the culture incubated at 37°C and 250 rpm for 4 hrs. The cells were pelleted by centrifugation at 5000 *g* for 30 minutes at 4°C and the supernatant mixed with 0.2 volumes of 20% (w/v) PEG-6000 in 2.5 M NaCl and left to stand for 1 hr at

4°C. The precipitate was pelleted by centrifugation at 5000 *g* for 20 minutes and the PEG-NaCl removed using a drawn-out Pasteur pipette. The viral pellet was resuspended in 500 µl of TE buffer and centrifuged for 5 minutes in a microfuge to remove any remaining cells. 200 µl of PEG-6000 in 2.5 M NaCl was added to the supernatant and the mixture left to stand at room temperature for 15 minutes. The precipitate was pelleted by centrifugation for 5 minutes in a microfuge, the PEG-6000 supernatant removed and the viral pellet resuspended in 500 µl of TE buffer. DNA was purified from this pellet by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was washed in cold (-20°C) 70% (v/v) ethanol, dried and resuspended in water to a final concentration of 1 µg/µl.

2.17.4 Mutagenesis Reaction

5 µl of single-stranded DNA template (1 µg/µl) was mixed with 2.5 µl of the phosphorylated mutant oligonucleotide (1.6 pmol/µl), 3.5 µl of buffer 1 and 6 µl of water. The mixture was placed in a water bath at 70°C for 3 minutes, before being transferred to a water bath at 37°C for 30 minutes to allow the oligonucleotide to anneal to the template DNA. The mutant DNA strand was synthesised and ligated by the addition of 5 µl of $MgCl_2$ solution, 19 µl of nucleotide mix, 6 µl of water, 6 units of DNA polymerase 1 (cloned Klenow fragment) and 6 units of T4 DNA ligase and incubation at 16°C overnight. 170 µl of water and 30 µl of 5 M NaCl were added to the reaction mix, which was loaded onto a nitrocellulose filter unit and centrifuged at 500 *g* for 10 minutes. Heteroduplex DNA passes straight through the filter while any single-stranded DNA binds to the filter. 100 µl of 500 mM NaCl was added to the filter to wash through any remaining double-stranded DNA and the centrifugation step repeated. The filtrate was ethanol precipitated and the pellet resuspended in 25 µl of buffer 2. 10 µl of this sample was digested with *Nci*I (5 units) in 65 µl of buffer 3 for 90 minutes at 37°C. This causes nicks to be introduced in the non-mutant strand but will not cut the mutant strand as this contains thionucleotides which are not recognised by *Nci*I. The non-mutant strand could then be removed by digestion with

exonuclease III, which degrades double-stranded DNA in a 3' to 5' direction from free 3' ends. 50 units of exonuclease III, 12 µl of 500 mM NaCl and 10 µl of buffer 4 were added to the sample, which was incubated at 37°C for 30 minutes. This allows the exonuclease III to remove approximately 3000 bases. The enzymes were inactivated by heating to 70°C for 15 minutes. The mutant strand was then used as a template for repolymerisation and ligation of the gapped DNA by the addition of 13 µl of nucleotide mix 2, 5 µl of $MgCl_2$ solution, 3 units of DNA polymerase I and 2 units of T4 DNA ligase, and incubation at 16°C for 3 hours. 20 µl of sample was used to transform competent TG1 cells which were plated on H-plates and incubated overnight at 37°C. Single-stranded DNA was isolated from plaques and sequenced to ensure that it contained the required mutation.

2.17.5 Reconstruction of the cDNA Encoding the Mutant Pre-33K

The complete 33K precursor was reconstructed in M13mp19 before being subcloned into pGEM-4Z for *in vitro* transcription and translation.

A phage supernatant containing the required mutant was prepared as described in section 2.17.3 and used to infect 250 ml of TY containing 2.5 ml of an overnight culture of JM109 cells. This was grown for 5 hrs at 37°C with agitation. Double stranded DNA was isolated from this culture as in section 2.14. The resulting DNA was digested with *Pst*I to release a 170 bp fragment of DNA and the linear vector containing the insert carrying the mutation isolated from an agarose gel (section 2.9) and treated with CIP to prevent re-ligation of the vector. p33K-2 vector was digested with *Pst*I to release a 1.1 kb fragment which was isolated from an agarose gel. This fragment was ligated into the vector previously isolated and the ligation mixture used to transform competent JM109 cells and plated out on L-amp plates. After incubation overnight at 37°C, plaques were picked from the plates and single-stranded DNA isolated. The DNA was sequenced to determine preparations containing the insert in the correct orientation and double-stranded DNA prepared. This DNA was digested with *Eco*RI, releasing a 1.3 kb fragment encoding the entire mutant 33K precursor

which was isolated from an agarose gel. The fragment was ligated into a pGEM-4Z vector previously linearised with *Eco*RI and used to transform competent JM109 cells, plated out on L-amp plates and incubated overnight at 37°C. Colonies from these plates were picked and plasmid DNA isolated for transcription. The DNA thus isolated was plasmid sequenced to ensure that it contained the expected mutation.

2.18 In Vitro *Transcription of Plasmid DNA*

2 µg of DNA was incubated with 15.5 µl of transcription premix (40 mM Tris-Cl pH 7.5, 6 mM $MgCl_2$, 2 mM Spermidine, 10 mM DTT, 500 µM rATP, rCTP, rUTP, 50 µM rGTP, 100 µg/ml nuclease free bovine serum albumin (BSA)), 1 unit/µl ribonuclease inhibitor, 500 µM m$_7$G(5')ppp(5')G (cap analogue) and 15 units of SP6 or T7 RNA polymerase. The mixture was incubated at 37°C for 30 minutes, rGTP added to a final concentration of 600 µM and the mixture further incubated for 30 minutes at 37°C.

2.19 Translation of RNA Transcripts in a Wheatgerm Lysate System

Wheatgerm lysate was provided by A. Hulford (Warwick), prepared as described by Roberts and Patterson (1973) and Anderson *et al.* (1983).

1 µl of transcription reaction mix containing the required RNA was mixed with 3.75 µl of wheatgerm lysate, 2.40 µl of translation premix (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)-KOH pH 7.6, 1 mM ATP, 8 mM creatine phosphate, 40 µg/ml creatine phosphokinase, 30 µg/ml Spermidine pH 7.0, 2 mM DTT, 20 µM GTP, 25 µM amino acid mix (from 5 mM stock excluding methionine)), 1 µl of [^{35}S]methionine 30 TBq/nmol (15 µCi/µl) and 4.35 µl of water and incubated at 27°C for 60 minutes. The products of the translation reaction were analysed by SDS-PAGE followed by fluorography.

2.20 Translation of RNA Transcripts in a Rabbit Reticulocyte Lysate System

The translation reaction was carried out according to the manufacturer's recommendations.

1 µl of transcription reaction mix containing the required RNA was mixed with 17.5 µl of rabbit reticulocyte lysate, 3.5 µl of water, 0.5 µl of ribonuclease inhibitor, 0.5 µl of 1 mM amino acids excluding methionine and 2 µl of [^{35}S]methionine 30 TBq/nmol (15 µCi/µl). The mixture was incubated at 30°C for 60 minutes and the reaction products analysed by SDS-PAGE and fluorography.

2.21 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Laemmli (1970)

2.21.1 Preparation of Mini Gels

Two glass plates, one plain and one notched, were combined with two plastic spacers to form a mould for a slab gel of 15.5 cm x 12 cm x 0.5 cm. 1% agarose was dissolved by microwaving and used to fill a plastic trough into which the gel mould was placed, the agarose forming a plug once set. 15% gels were prepared as below:

Resolving Gel

	volume	Final conc
Acrylamide/Bis-acrylamide (30%/0.8% (w/v))	10 ml	15%/0.4%
3 M Tris-Cl pH 8.8	2.5 ml	375 mM
H_2O	7.25 ml	
10% (w/v) SDS	200 µl	0.1%
10% (w/v) ammonium persulphate	83 µl	0.04%
TEMED	8.3 µl	

This solution was poured into the mould and overlaid with water-saturated butan-1-ol. After the gel had polymerised, the butan-1-ol was removed by washing with water and a stacking gel solution prepared as below:

Stacking Gel

	volume	Final conc.
Acrylamide/Bis-acrylamide (30%/0.8% (w/v))	2 ml	6%/0.16%
0.5 M Tris-Cl pH 6.8	2.5 ml	125 mM
H_2O	5.0 ml	
10% (w/v) SDS	100 µl	0.1%
10% (w/v) ammonium persulphate	50 µl	0.05%
TEMED	13 µl	

The stacking gel solution was then poured over the resolving gel, a well-former inserted and the gel left to polymerise. After polymerisation was complete, the well-former was removed carefully and the wells washed to ensure the removal of any remaining unpolymerised acrylamide solution. The gel was removed from the trough and assembled into a gel tank (Scieplas, U.K.) for electrophoresis. The tank reservoirs were filled with 1x running buffer (25 mM Tris-Cl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) and previously prepared samples were loaded into the wells. Electrophoresis was carried out for 2 hours at a constant current of 35 mA.

2.21.2 Preparation of Samples

Samples for electrophoresis were mixed with an equal volume of 2x protein sample buffer, boiled for 2 minutes and loaded onto the gel.

Protein Sample Buffer (2x)

	volume	final concentration
0.5 M Tris-Cl pH 8.0	2.5 ml	125 mM
H_2O	0.5 ml	
Glycerol	2.0 ml	20% (v/v)
10% (w/v) SDS	4.0 ml	0.4% (w/v)
2-mercaptoethanol	1.0 ml	10% (v/v)
Bromophenol blue	a few grains	

2.21.3 Coomassie Blue Staining of Gels

After electrophoresis the gel was removed from the gel plates and placed into stain solution for 20 minutes with shaking. The gel was then shaken in destain solution until the background was clear.

Stain Solution

Acetic Acid	7% (v/v)
Methanol	40% (v/v)
Coomassie Brilliant Blue R-250	0.5% (w/v)

Destain Solution

Acetic Acid	7% (v/v)
Methanol	40% (v/v)

2.21.4 Silver Staining of Gels

Silver staining is a highly sensitive method for staining gels and so was the preferred method of visualisation of protein profiles of column fractions.

After electrophoresis the gel was soaked overnight in 50% (v/v) methanol with three changes. The methanol was removed by washing the gel in water for 30 minutes and the gel then placed in silver stain solution for 15 minutes. Silver stain

solution was freshly prepared by mixing 1.4 ml of concentrated ammonia solution (35%) with 21 ml of 0.36% (w/v) NaOH. 4 ml of 0.2 g/ml silver nitrate was added dropwise with constant stirring and the solution made up to 100 ml with water. After washing with 500 ml of water for 10 minutes to remove the stain solution, the stain was developed by shaking the gel in developer solution freshly prepared by mixing 2.5 ml of 1% (w/v) citric acid and 0.3 ml of concentrated formaldehyde solution (40%) and making up to 500 ml with water. The gel was left in stain solution for 10-30 minutes or until the protein bands could be seen clearly. Further staining was prevented by shaking in destain solution as for Coomassie Blue staining.

2.21.5 Drying of Gels

After the appropriate staining, gels were placed between two sheets of cellophane and dried in an "Easy Breeze" gel dryer (Hoefer Scientific Instruments, U.S.A.) according to the manufacturer's instructions.

2.21.6 Fluorography

Radioactively labelled protein bands were visualised by fluorography. After destaining of the gel to fix the protein and to remove any radiolabelled amino acid not incorporated into protein, the gel was placed in "Amplify™" for 20 minutes prior to drying as described. The dried gel was placed into a light-proof cassette and exposed to X-ray film (Fuji, Japan). Films were developed under darkroom conditions using LX24 developer and fixed using Unifix, both used after diluting 1:4 with water.

2.22 Preparation of Pea Stromal Fraction

2.22.1 Growth of Pea Plants

Pea seeds (*Pisum sativum*, variety Feltham First, obtained from Booker Seeds, Nottingham) were grown in compost (Fisons Levington Multipurpose) for 12 days at

20°C +/- 2°C under a 12 hr photoperiod. Light intensity was approximately 50 $\mu E/m^2/sec$. The seeds were watered on alternate days with tap water.

2.22.2 Isolation of Chloroplasts

Method as described by Robinson and Barnett (1988).

Pea leaves were harvested and homogenised using a Polytron (Northern Media Supplies Ltd., Hull) at 75% full speed, 3x4 sec bursts in partially frozen sucrose isolation medium (SIM, 0.35 M sucrose, 25 mM HEPES-NaOH pH 7.6, 2 mM EDTA), 400 ml/100 g of leaves. The homogenate was filtered through 8 layers of muslin and centrifuged for 1 minute at 3000 *g* at 4°C to pellet the chloroplasts. The supernatant was discarded and the pellet washed with SIM.

2.22.3 Lysis of Chloroplasts

The chlorophyll concentration of the chloroplast pellet was determined by the method of Arnon (1949). Chloroplasts were resuspended to 1 mg/ml chlorophyll in 20 mM Tris-Cl pH 8.0 and placed on ice for 20 minutes. This causes lysis of the envelope membranes but the thylakoids remain intact. The suspension was centrifuged at 35,000 *g* for 20 minutes at 4°C to pellet the membranes, giving a stromal supernatant. The pellet was resuspended in 20 mM Tris-Cl pH 8.0 and again centrifuged at 35,000 *g* for 20 minutes at 4°C to extract any stroma remaining within the membrane fraction, and the two supernatants pooled.

2.23 Partial Purification of an SPP Activity from Pea Chloroplasts

In order to characterise further the SPP activity involved in the processing of chloroplast precursor proteins, SPP activity was partially purified from pea chloroplasts. All purification steps were performed at 4°C.

2.23.1 Ammonium Sulphate Fractionation of Pea Stromal Extract

A stromal extract was prepared from pea chloroplasts isolated from 800 g of pea leaves as described in section 2.22. 1 M Tris-Cl pH 8.0 was added to the extract to give a final concentration of 100 mM Tris-Cl pH 8.0. Ammonium sulphate was then added to the extract over 30 minutes to produce a 40% saturated solution and the extract left for 30 minutes to allow precipitation of protein. The sample was centrifuged at 30,000 *g* for 20 minutes to pellet the precipitated protein, which was discarded. Ammonium sulphate was added to the supernatant to 70% saturation over 30 minutes and again the extract left for 30 minutes before centrifugation at 30,000 *g* for 20 minutes. The protein precipitate was resuspended in 3 ml of 20 mM Tris-Cl pH 8.0.

2.23.2 Sephacryl S-300 Chromatography

The ammonium sulphate precipitated sample was loaded onto a Sephacryl S-300 gel filtration column (500 ml) pre-equilibrated with 20 mM Tris-Cl pH 8.0. Protein was eluted using 20 mM Tris-Cl pH 8.0, run at 40 ml/hr and 5 ml fractions collected using a Pharmacia (Sweden) Frac 100 collector. The optical density at 280 nm for each fraction was determined, fractions were assayed for cleavage of radiolabelled pre-PC (section 2.26) and protein in the fractions visualised by silver staining of SDS-PAGE gels.

2.23.3 Q-Sepharose Chromatography

The pooled sample from Sephacryl S-300 chromatography containing SPP activity was loaded onto a Q-Sepharose ion exchange column (50 ml) pre-equilibrated with 20 mM Tris-Cl pH 8.0. A 150 ml linear gradient of 0 mM NaCl, 20 mM Tris-Cl pH 8.0 - 500 mM NaCl, 20 mM Tris-Cl pH 8.0 was run at 40 ml/hr and 4 ml fractions were collected. Fractions were dialysed overnight against 20 mM Tris-Cl pH 8.0 to remove NaCl before assaying for SPP activity and visualisation of proteins by silver staining.

2.24 Partial Purification of an SPP Activity from Chlamydomonas reinhardtii

2.24.1 Growth of C. reinhardtii *Strain CC-400* cw-15

200 ml of tris/acetate/phosphate (TAP) medium was inoculated with *C. reinhardtii* cell wall-less mutant (strain CC-400 *cw-15*) mating type + and grown for 4 days with stirring at 20°C +\- 2°C under a 12 hr photoperiod. This culture was used to inoculate 4 l of TAP medium and this larger culture was grown under the same conditions with aeration for a further 5 days to a concentration of 0.6 - 1.0 x 10^7 cells/ml.

TAP medium 1 litre contains

Tris	2.42 g
TAP salts	25.00 ml
1 M potassium phosphate pH 7.0	0.38 ml
Hutner solution	1.00 ml
Glacial acetic acid	1.00 ml

TAP salts

NH_4Cl	15 g/l
$MgSO_4.7H_2O$	4 g/l
$CaCl_2.2H_2O$	2 g/l

Hutner Solution

Solutions were made up as follows:

EDTA	50 g in 250 ml boiling H_2O
$ZnSO_4.7H_2O$	22 g in 100 ml H_2O
H_3BO_3	11.4 g in 200 ml H_2O
$MnCl_2.4H_2O$	5.06 g in 50 ml H_2O
$CoCl_2.6H_2O$	1.61 g in 50 ml H_2O
$CuSO_4.5H_2O$	1.57 g in 50 ml H_2O
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.10 g in 50 ml H_2O
$FeSO_4.7H_2O$	4.99 g in 50 ml H_2O

All the solutions except EDTA were mixed, the mixture was brought to the boil and the EDTA solution added. The mixture was allowed to cool to 70°C and adjusted to pH 6.7 with 20% (w/v) KOH at 70°C. Water was added to a total volume of 1 litre, the flask stoppered with a cotton wool plug and left to stand for 2 weeks with occasional shaking. A rust brown precipitate formed, leaving a purple solution. The mixture was filtered to remove the precipitate and the solution stored at -20°C.

2.24.2 Preparation of Intact Chloroplasts from C. reinhardtii

Cells were harvested by centrifugation at 5,000 *g* for 5 minutes at 4°C, washed in 100 ml of 20 mM HEPES-KOH pH 7.5, and re-centrifuged to pellet the cells. Cells were resuspended in 50 mM HEPES-KOH pH 7.5, 2 mM EDTA, 1 mM $MgCl_2$, 300 mM sorbitol, 1% (w/v) BSA to a concentration of 0.3 mg/ml chlorophyll and passed through a 27 guage needle to break open the cells, leaving the chloroplasts intact. The suspension was centrifuged at 3,000 *g* for 10 minutes at 4°C to pellet the chloroplasts and the supernatant discarded.

2.24.3 Preparation of a Stromal Extract From C. reinhardtii *Chloroplasts*

Intact chloroplasts were resuspended to a concentration of 1 mg/ml chlorophyll in 20 mM Tris-Cl pH 8.0 and incubated for 20 minutes on ice to allow

lysis of the chloroplasts. The chloroplasts were then centrifuged at 30,000 *g* for 20 minutes at 4°C to pellet the membranes and the supernatant removed. The pellet was resuspended in 20 mM Tris-Cl pH 8.0, the centrifugation step repeated, and the stromal supernatants pooled.

2.24.4 Preparation of a Total C. reinhardtii *Soluble Cell Extract*

Attempts to isolate intact chloroplasts from *C. reinhardtii* produced a very low yield, and thus it was decided to use a total soluble cell extract as a starting material for purification of SPP activity. A wild-type *Chlamydomonas reinhardtii* strain (11/32b *mt*$^+$) was grown as described for the cell wall-less mutant and a 4 l culture harvested by centrifugation at 700 *g* for 20 minutes at 4°C. The cells were washed in 120 ml of 20 mM Tris-Cl pH 8.0, re-pelleted by centrifugation at 900 *g* for 20 minutes at 4°C and resuspended in 30 ml of 20 mM Tris-Cl pH 8.0. The cells were then passed twice through a French pressure cell (American Instrument Company) at 3400 p.s.i., breaking both cells and chloroplasts.The suspension was centrifuged at 900 *g* for 5 minutes at 4°C to pellet any cell debris and $MgCl_2$ was added to the supernatant to a final concentration of 5 mM. This supernatant was centrifuged at 8000 *g* for 10 minutes at 4°C to pellet the thylakoids, followed by centrifugation at 31,500 *g* for 30 minutes at 4°C to produce a soluble extract. This total soluble cell extract was filtered through a 2 micron filter before loading onto chromatography columns.

2.24.5 Q-Sepharose Chromatography

A Q-Sepharose anion exchange column (15 ml) was pre-equilibrated with 20 mM Tris-Cl pH 8.0 and *C. reinhardtii* total soluble extract was loaded onto the column. Protein was eluted using a 50 ml gradient of 0 mM NaCl, 20 mM Tris-Cl pH 8.0 - 500 mM NaCl, 20 mM Tris-Cl pH 8.0 at 20 ml/hr and 3 ml fractions collected. Fractions were assayed for cleavage of *C. reinhardtii* pre-SSU after dialysis against

20 mM Tris-Cl pH 8.0 to remove the NaCl and protein visualised by silver staining of SDS-PAGE gels.

2.24.6 Sephacryl S-300 Chromatography

Active fractions from a Q-Sepharose column were concentrated by ammonium sulphate precipitation, loaded onto a Sephacryl S-300 gel filtration column (200 ml) pre-equilibrated with 20 mM Tris-Cl pH 8.0 and protein eluted using 20 mM Tris-Cl pH 8.0 at 20 ml/hr. 3.5 ml fractions were collected and assayed as described.

2.24.7 Hydroxylapatite Chromatography

Sephacryl S-300 active fractions were loaded onto a 7.5 ml hydroxylapatite column pre-equilibrated with 10 mM sodium phosphate buffer pH 8.0 and 2 ml fractions eluted at 20 ml/hr with a 30 ml gradient of 10 - 100 mM sodium phosphate buffer pH 8.0 and assayed as described.

2.25 Preparation of Maize Mitochondrial Extract

Maize mitochondria were prepared by A. Liddell (Oxford) from 3-4 day old etiolated maize coleoptiles grown at 27°C and purified through a sucrose density gradient as described by Leaver, Hack and Forde (1983).

10 mg of mitochondria were suspended in 1 ml of 30 mM Tris-Cl pH 8.0 containing 1% (v/v) Triton X-100. The suspension was incubated for 20 minutes on ice to allow lysis of the mitochondria and centrifuged for 30 minutes in a microfuge to pellet the membrane fraction. The supernatant was removed and assayed for MPP activity.

2.26 In Vitro *Assay for Processing of Precursor Proteins*

For each assay the precursor was synthesised *in vitro* in a wheatgerm or rabbit reticulocyte lysate system.

2.26.1 SPP

1 µl of translation mixture was mixed with 20 µl of SPP preparation containing 0.1% Triton X-100 and 1 mM phenyl methyl sulphonyl fluoride (PMSF). This mixture was incubated at 27°C for 90 minutes.

2.26.2 MPP

A reaction mix contained the following components:

Precursor protein	1.00 µl
MPP (0.5 mg/ml)	0.25 µl
PEP (0.3 mg/ml)	0.50 µl
PMSF (200 mM)	0.30 µl
DTT (100 mM)	0.30 µl
$MnCl_2$ (25 mM)	0.40 µl
30 mM Tris-Cl pH 8.0	17.05 µl
Triton X-100	0.20 µl

This mix was incubated at 30°C for 45 minutes. MPP and PEP were provided by Dr M. Arretz (Munich).

2.26.3 Maize Mitochondrial Extract

A reaction mix was prepared as follows:

Precursor protein	1.00 µl
Mitochondrial extract	17.80 µl
PMSF (200 mM)	0.30 µl
DTT (100 mM)	0.30 µl
$MnCl_2$ (25 mM)	0.40 µl
Triton X-100	0.20 µl

This mix was incubated at 30°C for 90 minutes.

After incubation with the appropriate enzyme, each reaction was stopped by boiling in an equal volume of protein sample buffer. Products of the reaction were analysed by SDS-PAGE followed by fluorography.

2.27 Edman Degradation of Processed Forms of Chloroplast or Mitochondrial Proteins

Precursor protein was synthesised in a wheatgerm or rabbit reticulocyte lysate translation system in the presence of a radiolabelled amino acid (Amersham, U.K.) at 1 µCi/µl of translation mixture as described for [^{35}S] methionine (see sections 2.19 and 2.20). 15-30 µl of translation mixture was incubated with SPP or MPP as described in section 2.26 to generate the processed form of the protein. The mixture was resolved on an SDS-PAGE gel according to the conditions described in Applied Biosystems User Bulletin No. 25. The gel was then blotted onto Immobilon membrane (Millipore, U.K.) and the band corresponding to the processed protein identified by counting slices for radioactivity. The band was excised and placed in the cartridge block of an Applied Biosystems model 470a protein sequencer equipped with a 120a on-line phenylthiohydantoin analyzer. Fractions from each cycle were counted for radioactivity. Radiosequencing was performed by B. Dunbar (Aberdeen).

2.28 Isolation of Intact Chloroplasts for the Uptake of Proteins

2.28.1 Isolation of Intact Chloroplasts

Chloroplasts were isolated as described previously from 100 g of pea leaves and the pellet resuspended in 4 ml of sucrose resuspension medium (SRM, 50 mM HEPES-KOH pH 8.0, 0.33 M sorbitol). A 4 ml 35% Percoll pad in SRM was prepared and the chloroplast suspension loaded carefully onto the pad. The sample was centrifuged at 2500 *g* for 7 minutes at 4°C. This causes intact chloroplasts to be pelleted leaving broken chloroplasts on the top of the pad. The supernatant containing the broken chloroplasts was discarded and the pellet washed in SRM. The chlorophyll concentration was determined and the chloroplasts resuspended in SRM to a concentration of 1 mg/ml chlorophyll.

2.28.2 Import of Precursors into Intact Chloroplasts

The uptake of *in vitro* translation products into isolated chloroplasts was assayed using a procedure modified from that of Chua and Schmidt (1978). Incubation mixtures contained the following, with all solutions made up in SRM:

Intact chloroplasts	55 µl
200 mM methionine	10 µl
100 mM ATP	7 µl
200 mM $MgCl_2$	3.5 µl

This mixture was pre-incubated in an illuminated water bath (300 $\mu E/m^2/sec$) at 25°C to allow the ATP to enter the chloroplasts. 5-10 µl of *in vitro* translation mixture was added to the incubation mixture and the tube returned to the water bath for 20 minutes with intermittent shaking. The chloroplasts were washed in 5 ml of SRM and re-isolated by centrifugation at 2500 *g* for 2 minutes at 4°C. The pellet was resuspended in 60 µl of SRM. 30 µl was removed and stored at -80°C and to the remainder, 3 µl of 2 mg/ml thermolysin was added. The sample was incubated on ice for 40 minutes and 1 ml of SRM/10 mM EDTA added. Chloroplasts were pelleted by

centrifugation and resuspended in 30 µl of SRM/10 mM EDTA. Samples were analysed by SDS-PAGE and fluorography.

2.29 Localisation of Protein Imported into Intact Chloroplasts

To determine whether an imported protein was located in the stroma or the thylakoid, an import reaction was carried out as above but with double the quantities. After incubation in the light bath for 20 minutes to allow import, chloroplasts were washed with 5 ml of SRM. The chloroplast pellet was resuspended in 120 µl of SRM and 30 µl removed and stored at -80°C. 30 µl was treated with thermolysin as described in section 2.28. The remaining 60 µl was centrifuged for 30 seconds in a microfuge and the pellet resuspended in 10 mM Tris-Cl pH 7.6/5 mM $MgCl_2$. The suspension was placed on ice for 5 minutes to allow lysis of the chloroplasts and centrifuged for 1 minute in a microfuge to pellet the thylakoids. The stromal supernatant was removed and placed at -80°C and the thylakoids washed with 10 mM Tris-Cl pH 7.6/5 mM $MgCl_2$ and resuspended in 60 µl of 10 mM Tris-Cl pH 7.6/5 mM $MgCl_2$. 30 µl was removed and stored at -80°C. The final 30 µl was incubated with 3 µl of 2 mg/ml thermolysin for 40 minutes on ice and washed with 1 ml of 10 mM Tris-Cl pH 7.6/5 mM $MgCl_2$/10 mM EDTA. The thylakoid pellet was resuspended in 30 µl of 10 mM Tris-Cl pH 7.6/5 mM $MgCl_2$/10 mM EDTA. All samples were analysed by SDS-PAGE followed by fluorography.

2.30 Western Blotting

Proteins for Western blotting were separated by SDS-PAGE and the gel was placed onto an Ancos (Denmark) semi-dry transblotting apparatus in contact with a nitrocellulose membrane. On passing an electric current through the apparatus the proteins were transferred onto the nitrocellulose membrane. Blotting was carried out for 1 hour at a constant current of 0.8 mA/cm^2 of gel using a transfer buffer of 6.25 mM Tris-Cl pH 8.3, 48 mM glycine, 0.025% (w/v) SDS, 20% (v/v) methanol. After blotting, the gel was Coomassie blue stained and the nitrocellulose filter was placed

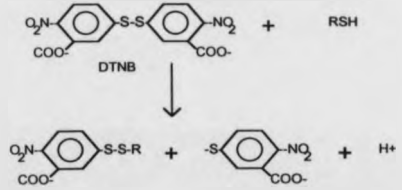
into phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4)) containing 0.1% (v/v) Tween 20 and 10% (w/v) "Marvel" dried milk for 90 minutes to prevent non-specific binding of antibodies to the membrane. The membrane was washed three times in PBS containing 0.1% Tween 20 for 5 minutes and incubated overnight with a polyclonal antibody specific to the *Neurospora crassa* β subunit of the F_1 ATPase (provided by Prof W. Neupert, Munich) in 5 ml of PBS containing 0.1% (v/v) Tween 20, 2% (w/v) "Marvel". The membrane was then washed three times in PBS containing 0.1% (v/v) Tween 20 and incubated in 5 ml of PBS containing 0.1% (v/v) Tween 20 and 16.5 µl of biotinylated protein A for 45 minutes. The membrane was washed three times in PBS containing 0.3% (v/v) Tween 20 and three times in PBS containing 0.1% (v/v) Tween 20 before being incubated with 5 ml of PBS containing 0.1% (v/v) Tween and 16.5 µl of biotinylated Streptavidin-horseradish peroxidase complex for 45 minutes. The membrane was then washed three times in PBS containing 0.1% (v/v) Tween 20 and once in PBS. The proteins were visualised using an Amersham ECL (enhanced chemi-luminescence) kit according to the manufacturers instructions, filters were exposed to X-ray film for between 10 seconds and 30 minutes and the film developed as for SDS-PAGE gels.

2.31 Assay for Citrate Synthase Activity

Based on the method described by Srere (1969). The reaction catalysed by citrate synthase is:

$$\text{Acetyl-CoA} + \text{oxaloacetate}^{2-} + H_2O \Leftrightarrow \text{citrate}^{3-} + \text{CoASH} + H^+$$

The activity is followed by measuring the appearance of the free SH group in CoASH using 5,5'-dithio-bis-(2-nitrobenzoic) acid (DTNB).



The mercaptide ion released has a strong absorbance at 412 nm which can be used to monitor the reaction spectrophotometrically.

10 µl of maize mitochondrial matrix preparation containing 0.1 mg of protein or 10 µl of pea total extract or chloroplast preparation containing 0.01 mg of chlorophyll were added to 810 µl of water and centrifuged for 10 minutes in a microfuge to pellet any membranes present. To this mixture was added 100 µl of 0.4 mg/ml DTNB in 1 M Tris-Cl pH 8.0 and 10 µl of 10 mg/ml acetyl coenzyme A pH 5.0. The absorbance at 412 nm was measured for 2 minutes to determine the acetyl coenzyme A hydrolase activity. 50 µl of 1.32 mg/ml oxaloacetic acid (OAA) was added and the absorbance recorded for a further 5 minutes. The change in absorbance per minute before adding OAA was subtracted from the change in absorbance per minute after adding OAA to determine the net rate. This rate is then equivalent to 13.6 x µmol of coenzyme A produced per minute by the amount of enzyme added.

2.32 Precipitation of Protein using Trichloroacetic Acid

The solution containing the protein to be precipitated was mixed with trichloroacetic acid (TCA) to a final concentration of 6% and incubated for 20 minutes on ice. The protein was pelleted by centrifugation in a microfuge for 10 minutes, washed twice with 400 µl of acetone and resuspended in an appropriate volume of sample buffer.

2.33 Bio-Rad Protein Assay

When protein binds to Coomassie Brilliant Blue G-250, the absorbance maximum of the Coomassie solution shifts from 465 nm to 595 nm, and this provides a useful method for determining protein concentration in solution.

2.33.1 Standard Assay

Bio-Rad reagent was diluted five-fold and filtered through Whatman No. 1 paper before use. Lysozyme was used as a protein standard for the preparation of a standard curve of protein concentration. Appropriate dilutions were made of the lysozyme between approximately 0.1 and 1.5 mg/ml. 0.1 ml of the standards or the samples to be measured were placed in test tubes and 5 ml of the Bio-Rad reagent added. The samples were left to stand for 10 minutes at room temperature and the OD_{595} measured against a blank consisting of 0.1 ml of buffer and 5 ml of reagent. A graph was plotted of OD_{595} against the concentration of the standards and the concentration of the samples read from the standard curve.

2.33.2 Microassay

For very low protein concentrations, a modified procedure was used for the determination of protein concentration. Dilutions of a lysozyme protein standard were made to between 1 and 25 µg/ml. 0.8 ml of each standard and the samples to be measured were mixed with 0.2 ml of Bio-Rad reagent and left for 10 minutes at room temperature. The OD_{595} was measured for each sample against a blank consisting of 0.8 ml of buffer and 0.2 ml of dye reagent and the OD_{595} for each of the standards plotted against protein concentration. The concentrations of the samples were then read from the standard curve.

2.34 Acetone Precipitation of Protein

Samples to be precipitated (10 µl) were mixed with 1 ml of 80% (v/v) acetone, incubated on ice for 10 minutes to allow all of the protein to be precipitated, centrifuged for 10 minutes in a microfuge to pellet the protein and the supernatant discarded. The pellet was washed with 200 µl of ether to remove the acetone and allowed to dry. The protein pellet was resuspended in 100 µl of 20 mM Tris-Cl pH 8.0 and the protein concentration determined as described in section 2.33.

2.35 Suppliers

All reagents used were of analytical grade if available and were obtained from the following sources, unless indicated otherwise in the text:

Aldrich (U.K.)

Tween 20.

Amersham (U.K.)

Amplify™, [α-^{35}S]dATP, biotinylated protein A, Streptavidin-horseradish peroxidase complex, T4 DNA ligase.

BDH (U.K.)

Acetone, ammonium persulphate, bromophenol blue, butan-1-ol, calcium chloride, chloroform, cobalt chloride, ethanol, ether, formaldehyde, HEPES, hydroxyquinoline, isopropanol, magnesium sulphate, methanol, PEG-6000, potassium acetate, SDS, Tris, triton X-100.

Bio-Rad Laboratories (U.S.A.)

Protein assay dye reagent concentrate.

Boehringer Mannheim (U.K.)

ATP, creatine phosphate, creatine phosphokinase, DTT, M13mp19 bacteriophage, thermolysin.

BRL (U.S.A.)

SP6 and T7 RNA polymerase, urea.

Calbiochem (U.S.A.)

Hydroxylapatite.

Difco (U.S.A.)

Agar, tryptone.

Fisons (U.K.)

Acetic acid, acrylamide, ammonia solution, ammonium chloride, ammonium sulphate, copper sulphate, glycine, iron sulphate, magnesium chloride, β-mercaptoethanol, phenol, potassium chloride, potassium dihydrogen orthophosphate, potassium hydroxide, sodium acetate, sodium chloride, disodium hydrogen orthophosphate, sodium hydroxide, sucrose.

Johnson Matthey (U.K.)

Silver nitrate.

Kodak (U.K.)

Bis-acrylamide, LX24 developer, Unifix.

May and Baker (U.K.)

Ammonium molybdate, zinc sulphate.

Northumbria Biologicals (U.K.)

Calf intestinal alkaline phosphatase.

Oxoid (U.K.)

Yeast extract.

Pharmacia (Sweden)

BSA, cap analogue, Percoll, Q-Sepharose, restriction enzymes, Sephacryl S-300, T4 polynucleotide kinase.

Prolabo (France)

Boric acid, EDTA, glycerol.

Promega (U.S.A.)

pGEM-4Z, rabbit reticulocyte lysate, ribonuclease inhibitor, SP6 and T7 primers, transcription premix.

Sigma (U.K.)

Acetyl coenzyme A, agarose, amino acids, ampicillin, citric acid, Coomassie blue, DTNB, ethidium bromide, formamide, IPTG, lysozyme, oxaloacetic acid, PMSF, RNase A, sorbitol, TCA, TEMED, x-gal, xylene cyanole FF.

CHAPTER 3 - PURIFICATION OF A STROMAL PROCESSING PEPTIDASE ACTIVITY FROM *PISUM SATIVUM*

3.1 Introduction

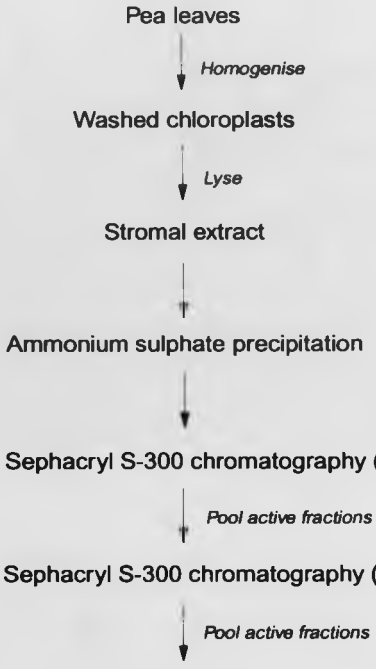
Purification of SPP from pea (*Pisum sativum*) chloroplasts was initially attempted with the aims of producing a highly active preparation in order to characterise the enzyme further and of identifying the band on a silver-stained gel corresponding to the enzyme. The approach taken was to improve the protocol described previously by Robinson and Ellis (1984) and Musgrove *et al.* (1989). At the beginning of this project, no protocol was available for purifying the enzyme to homogeneity.

3.2 Partial Purification of Pea SPP Activity

A stromal extract was prepared from 500 g (fresh weight) of peas as described in section 2.22. The major protein in this extract was rubisco and so the first steps in the purification described by Robinson and Ellis (1984) were repeated in order to remove as much of the rubisco as possible (figure 13). A 40-70% ammonium sulphate fractionation step was performed to concentrate the sample, whilst removing a small number of proteins and so providing a slight purification (section 2.23.1), and the sample was divided into two halves and each half then chromatographed on a column (2.5 x 100 cm) of Sephacryl S-300 high resolution resin (section 2.23.2). The Sephacryl S-300 high resolution resin is an equivalent material to the Sephacryl S-300 superfine originally used. SPP activity was assayed using *Silene pratensis* pre-PC as a substrate as described in section 2.26, with the results visualised by SDS-PAGE followed by fluorography using Amplify. The amount of protein in each fraction was estimated by measuring the absorbance at 280 nm and using a Bio-Rad protein assay. The fractions containing SPP activity immediately after the peak of absorbance at 280 nm corresponding to rubisco were pooled and again concentrated by ammonium

Figure 13 *Strategy for the purification of SPP activity from pea chloroplasts*

Activity was purified and assayed as described in Materials and Methods


Pea leaves
Homogenise
Washed chloroplasts
Lyse
Stromal extract
Ammonium sulphate precipitation
Sephacryl S-300 chromatography (1)
Pool active fractions
Sephacryl S-300 chromatography (2)
Pool active fractions
Q-Sepharose chromatography

sulphate precipitation. This sample was rechromatographed on a Sephacryl S-300 high resolution column and again the active fractions eluting after the peak of rubisco were pooled. Approximately 50% of the rubisco protein can be removed in each of the gel filtration steps, thus removing a large proportion of the rubisco in total.

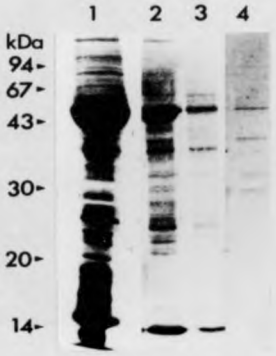
The next step in the original protocol was ion-exchange chromatography using a diethylaminoethyl (DEAE)-Sephacel column. This step was replaced by another anion-exchange step. The pooled fractions from the Sephacryl S-300 column were chromatographed on a Q-Sepharose Fast Flow column (1.5 x 25 cm; section 2.23.3) and eluted using a 150 ml linear gradient of 20 mM Tris-Cl pH 8.0 - 20 mM Tris-Cl pH 8.0, 500 mM NaCl. This material has a functional group of $-CH_2N^+[CH_3]_3$, which gives a sharper peak of activity than the DEAE-Sephacel used previously and the SPP activity purified in this way is free from other detectable protease activities. A silver-stained gel showing the proteins remaining after each purification step is shown in figure 14. Active fractions from the purification procedure described so far were used for all subsequent experiments involving a partially-purified pea SPP preparation.

3.3 Other Approaches to Purification

In the partial purification of stromal peptidases described by Musgrove *et al.*, (1989), fractionation involved DEAE-Sephacel chromatography followed by phenyl-Sepharose chromatography, with the aim of separating SPP from a range of other stromal endopeptidases which will also degrade chloroplast precursor proteins in an *in vitro* assay. Phenyl-Sepharose was therefore chosen as a candidate for the next step in purification. However, although this material gave a good resolution of proteins and a sharp peak of activity when used as an earlier step in the purification procedure, it did not result in a significant purification when used as a final step and much of the SPP activity of the initial sample was lost after the number of purification steps required. Several other column matrixes were also tested for use in purification steps, including a Superdex S-300 gel filtration column and immobilised affinity dye resins.

Figure 14 *Analysis of the purification of SPP activity from pea chloroplasts*

SDS-polacrylamide gel of 20 µl of a pea stromal extract (lane 1), eluate after a first Sephacryl S-300 chromatography step (lane 2), eluate after a second Sephacryl S-300 chromatography step (lane 3) and eluate after Q-Sepharose chromatography (lane 4). Each sample contains pooled fractions containing SPP activity, with protein visualised by silver-staining. Sizes of molecular weight markers are indicated along the side.


1
2
3
4
kDa
94
67
43
30
20
14

Fast protein liquid chromatography (FPLC) columns were also tested, in particular a Mono Q anion exchange column, a Mono S cation exchange column and a phenyl Superose hydrophobic interaction column, under a variety of running conditions (performed at Schering Agrochemicals, Saffron Walden). No significant progress in the purification was achieved with any of the purification steps attempted and so further purification of this enzyme was deemed to be impractical. A purification procedure for this enzyme was later published by Oblong and Lamppa (1992) involving a specific affinity column consisting of immobilised recombinant pre-LHCP.

CHAPTER 4 - DETERMINATION OF THE SPP CLEAVAGE SITE OF THREE THYLAKOID LUMEN PROTEINS

4.1 Introduction

Stromal proteins such as rubisco SSU contain N-terminal presequences which have been shown to contain the targeting information for translocation of the precursor across the double envelope membranes of the chloroplast (Van den Broeck *et al.*, 1985). The presequence is removed upon import into the chloroplast by SPP to produce the mature protein. Thylakoid lumen proteins also contain an N-terminal presequence, which directs import into the thylakoid lumen according to the two step import model (Hageman *et al.*, 1986; Smeekens *et al.*, 1986). The ETD was shown to contain the targeting information for translocation across the envelope membranes and so to be functionally equivalent to the presequences of stromal proteins (Hageman *et al.*, 1990). This domain is thought to be removed by the same enzyme responsible for removing the presequence from stromal proteins, although as yet there is no direct evidence for this. The TTD then directs translocation across the thylakoid membrane into the lumen where the remainder of the presequence is removed by TPP (Hageman *et al.*, 1986; James *et al.*, 1989; Kirwin *et al.*, 1989). The presequences of several lumenal precursors were compared by von Heijne *et al.* (1989) and shown to have two features in common: the presence of small, uncharged residues (usually alanine) at the -3 and -1 positions preceded by a stretch of hydrophobic residues. These features also occur in signal sequences directing proteins into the ER lumen in eukaryotes or periplasm in bacteria and TPP has been shown to be a signal-type peptidase with a similar cleavage specificity to signal peptidase, LEP and possibly mitochondrial IMP 1 (Halpin *et al.*, 1989; Shackleton and Robinson, 1991; Dalbey and von Heijne, 1992). To understand further the features of the ETD and TTD, the SPP cleavage site dividing the two must be determined, as this enables direct comparisons of the domains with the presequences of stromal proteins or with signal peptides.

The site of SPP cleavage of many stromal protein presequences has been deduced by N-terminal sequencing of the mature protein isolated from plant tissue and these cleavage sites appear to show very little primary structure similarity (von Heijne *et al.*, 1989). However, one problem with this approach is that the stroma contains several aminopeptidases (Liu and Jagendorf, 1986) which may remove residues from the N-terminus of imported proteins, and so the N-terminal residue in the isolated mature protein may not be that produced by SPP cleavage. The site of SPP cleavage for thylakoid lumen proteins is more difficult to estimate, as *in vivo* the intermediate forms do not exist for significant lengths of time, being rapidly converted to the mature form on entry into the thylakoid lumen, and it is impossible to deduce from cDNA sequence data where the SPP cleavage sites are located.

The aim of this part of my work was therefore to determine the sites of processing by SPP of three thylakoid lumen precursor proteins, 23K and 33K from wheat and *Silene pratensis* PC. This provides information on two aspects of the presequences; firstly, on the residues around the SPP cleavage site and secondly, on the extent of the ETD and TTD within the presequences. The experimental approach was to produce intermediate-sized proteins by incubating radiolabelled precursor proteins produced by *in vitro* transcription and translation with partially purified SPP. SPP cleaves very specifically with no evidence of incorrect processing of any precursor (Robinson and Ellis, 1984; Musgrove *et al.*, 1989) and the intermediates produced comigrate with stromal intermediates generated during protein import into intact chloroplasts (Hageman *et al.*, 1986; James *et al.*, 1989). These intermediates were subjected to automated Edman degradation and the SPP cleavage sites deduced from the pattern of release of radiolabelled amino acids. All Edman degradations were carried out by B. Dunbar (Aberdeen).

4.2 Determination of the SPP Cleavage Site in the Wheat 23K Presequence

Radiolabelled wheat pre-23K was synthesised by *in vitro* transcription and translation of plasmid p23K-1 (James *et al.*, 1989), a plasmid containing the full length cDNA encoding the wheat 23K precursor, in a wheatgerm lysate system in the presence of [^{3}H]lysine. The tritiated precursor protein was processed to the intermediate size by incubation with partially purified pea SPP. The precursor and intermediate were separated by SDS-PAGE and the intermediate form was subjected to automated Edman degradation as described. The fractions produced from each cycle were counted for ^{3}H radioactivity and the results shown in figure 15. A single peak of radioactivity was released at cycle 3 of the degradation. Apart from a lysine 7 residues before the terminal (TPP) cleavage site, the wheat presequence contains lysine residues only at positions 34 and 37 (James and Robinson, 1991). Cleavage by SPP must therefore take place immediately after lysine 34 for a single lysine residue (lysine 37) to emerge in cycle 3 of the sequencing run. This means that SPP processes pre-23K between a lysine residue and an alanine residue.

4.3 Determination of the SPP Cleavage Site in the Wheat 33K Presequence

Radiolabelled wheat pre-33K was synthesised by *in vitro* transcription and translation of plasmid p33K-2 (James *et al.*, 1989), a plasmid containing a full length cDNA encoding pre-33K, in a wheatgerm lysate system in the presence of [^{3}H]leucine or [^{3}H]phenylalanine. The protein was cleaved to the intermediate size by incubation with partially purified SPP and the intermediate separated from the precursor by SDS-PAGE. Edman degradation and counting were carried out as for i-23K. Degradation of the [^{3}H]leucine-labelled intermediate produced a single peak of radioactivity at cycle 14 (Figure 16B), whereas degradation of the [^{3}H]phenylalanine-labelled intermediate released a single peak at cycle 2 (Figure 16C). Comparison with the deduced protein sequence determined from the cDNA sequence (Meadows *et al.*, 1991) shows that to produce this pattern of release of radioactivity, SPP must have cleaved after arginine at position 31 in the presequence, as there are no other sites

Figure 15 *Identification of the SPP cleavage site within the wheat pre-23K presequence*

Panel A

Sequence of the complete presequence of wheat pre-23K. The arrow denotes the deduced site of cleavage by SPP. The residue radiolabelled for the Edman degradation analysis is underlined. + and - denote the charges on residues in the presequence.

Panel B

[^{3}H]lysine-labelled pre-23K was generated by *in vitro* transcription and translation and incubated with SPP to generate the intermediate form. This intermediate was subjected to Edman degradation and counts determined for each cycle.

A


E.T. MASTSCFLHQSTARLAASARPAPAVGRTQLFVVCK
T.T. AQKNDEAASDAAVVTSRRAALSLLAGAAAIAVKVSPAAA

B

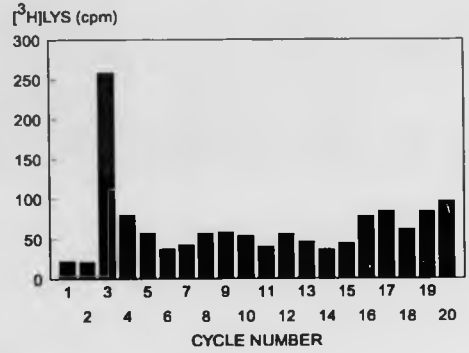

[3H]LYS (cpm)
300
250
200
150
100
50
0
1 3 5 7 9 11 13 15 17 19
2 4 6 8 10 12 14 16 18 20
CYCLE NUMBER

Figure 16 *Identification of the SPP cleavage site within the wheat pre-33K presequence*

Panel A

Sequence of the complete presequence of wheat pre-33K. The arrow denotes the deduced site of cleavage by SPP. Residues radiolabelled for the Edman degradation analysis are underlined. Charges on the amino acid residues in the presequence are indicated.

Panel B

[^{3}H]phenylalanine-labelled pre-33K was generated by *in vitro* transcription and translation and incubated with SPP to generate the intermediate form. This was subjected to Edman degradation and counts determined for each cycle.

Panel C

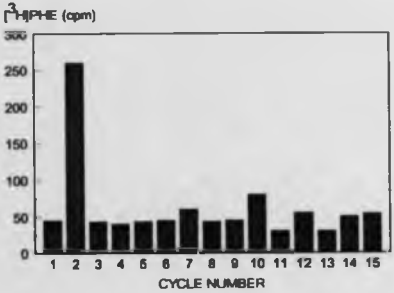
As panel B but the pre-33K was labelled with [^{3}H]leucine.

A

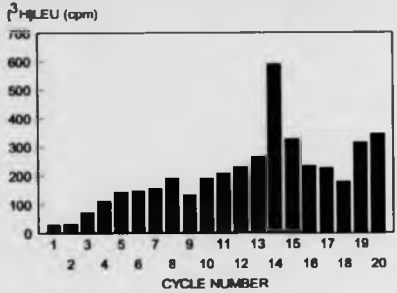
E.T. MAASLQAAATVMPAKIGGRASSARPSSHVAR

T.T. AEGVDAGARITCSLQSDIREVASKCADAAKMAGFALATSALLVSGATA

B



C



within the 33K presequence compatible with this data. SPP therefore cleaves in this case between an arginine and an alanine residue.

4.4 Determination of the SPP Cleavage Site in the Silene pratensis *Plastocyanin Presequence*

Radiolabelled pre-PC was generated by *in vitro* transcription and translation in a wheatgerm system as described (Hageman *et al.*, 1986) in the presence of [^{3}H]lysine or [^{3}H]leucine and the intermediate form produced by incubation with SPP. Separation of the intermediate and Edman degradation were as described for i-23K and i-33K. For the [^{3}H]lysine-labelled sample, a single peak of radioactivity was observed at cycle 4 of the degradation, indicating that cleavage must occur after lysine 41 of the presequence (Figure 17B). The [^{3}H]leucine-labelled intermediate produced a small peak of radioactivity at cycle 3 (Figure 17C), which is inconclusive on its own but confirms the result obtained for the [^{3}H]lysine sample, that SPP cleaves pre-PC after lysine 41 (Smeekens *et al.*, 1985). Previous work (Hageman *et al.*, 1986; Smeekens *et al.*, 1986) has shown that SPP cleaves pre-PC shortly after methionine 37, which is also confirmed by these data. The SPP cleavage site in *Silene pratensis* pre-PC is therefore between a lysine residue and an alanine residue.

4.5 Comparison of SPP Cleavage Sites

A comparison of the SPP cleavage sites of many stromal proteins has shown very little primary structure similarity around this site (von Heijne *et al.*, 1989). The microsequencing data described here provide information on the cleavage sites recognised by SPP in thylakoid lumen proteins. One striking feature of the cleavage sites identified is that in each case cleavage occurs between a positively charged residue and an alanine residue. This suggests that these features are important for recognition and/or cleavage of the presequences by SPP. However, although stromal protein presequences usually contain a positively charged residue close to their

Figure 17 ***Identification of the SPP cleavage site within the*** Silene pratensis ***pre-PC presequence***

Panel A

Sequence of the complete presequence of *Silene pratensis* pre-PC. The arrow denotes the deduced site of cleavage by SPP. Residues radiolabelled for the Edman degradation analysis are underlined and charges on the residues indicated.

Panel B

[^{3}H]lysine-labelled pre-PC was generated by *in vitro* transcription and translation and incubated with SPP to generate the intermediate form. This intermediate was subjected to Edman degradation and counts determined for each cycle.

Panel C

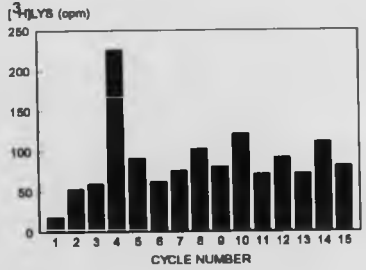
As for panel B but the pre-PC was labelled with [^{3}H]leucine.

A

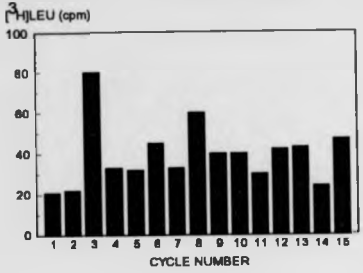
E.T. MATVTSSAAVAIPSFAGLKASSTTRAATVKVAVATPRMSIK

T.T. ASLKDVGVVVAATAAAGILAGNAMA

B



C



cleavage site, it is rarely in the position immediately before the site (von Heijne *et al.*, 1989). There are three possible explanations for this:

1. The cleavage motif in the thylakoid lumen proteins is a coincidence due to the small sample size.
2. A different enzyme is responsible for cleavage of precursor proteins destined for the thylakoid lumen or the stroma.
3. N-terminal residues are removed from stromal proteins after SPP cleavage, as described.

The third option is unlikely, as work by Clark and Lamppa (1991) has shown that altering the positively charged residues close to the SPP cleavage site in rubisco SSU and rubisco activase, both stromal proteins, has little effect on processing by SPP upon import into the chloroplast *in vitro*. However, relatively small differences in rate of cleavage would probably not be detected in this assay, so this does not rule out the possibility that the presence of the positive charge affects the efficiency of cleavage.

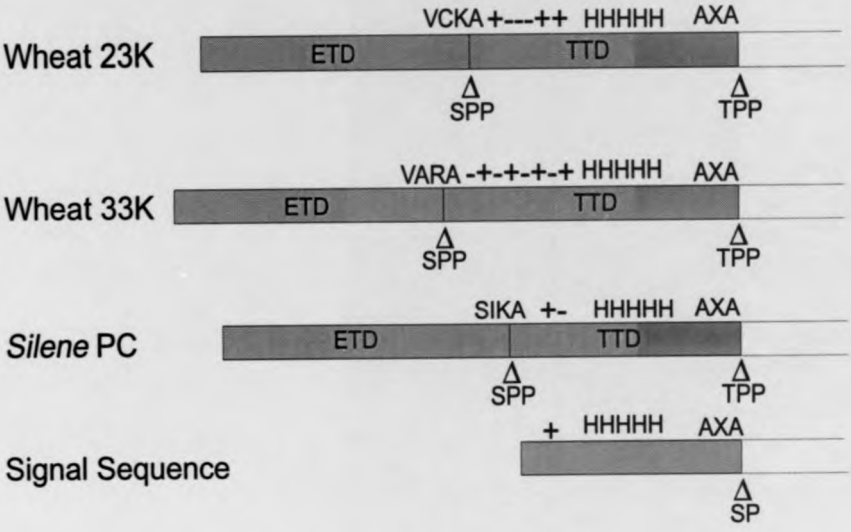
Except for the residues immediately adjacent to the cleavage sites, no primary structure motifs can be identified which may serve as a recognition signal for the enzyme and no common secondary structure can be predicted reliably at these sites. However, the radiosequencing results demonstrate the very high degree of reaction specificity demonstrated by SPP, as SPP clearly cleaves these proteins at a single site, as is evident in the sequencing runs where the background level of radioactivity was low (*eg* see Figure 16B). The structural requirements for a protein to contain a site recognised by SPP therefore remain obscure.

4.6 Delineation of Envelope Transit and Thylakoid Transfer Signals

The delineation of the envelope transit and thylakoid transfer domains of the thylakoid lumen proteins described here enables their comparison with prokaryotic and eukaryotic signal peptides (Figure 18). An analysis of signal peptides (von Heijne, 1985, 1986) has shown that they have several common features: they are

Figure 18 *Comparison of thylakoid lumen protein presequences and a typical signal sequence*

The structural features of the three thylakoid lumen protein presequences studied and a typical signal sequence are shown. All of the thylakoid transfer domains, in common with the signal sequence, contain a hydrophobic core region (HHHHH) and small, uncharged residues at the -3 and -1 positions (AXA). The differences in the domains can also be seen: thylakoid transfer domains tend to be longer and more highly charged than signal sequences. The sites of cleavage by SPP, TPP and SP are indicated by arrows.


VCKA +---++ HHHHH AXA
Wheat 23K
ETD
TTD
SPP
TPP
VARA -+-+-+-+ HHHHH AXA
Wheat 33K
ETD
TTD
SPP
TPP
SIKA +- HHHHH AXA
Silene PC
ETD
TTD
SPP
TPP
+ HHHHH AXA
Signal Sequence
SP

usually around 20 residues in length, although some are much longer, they have small, uncharged residues at the -1 and -3 positions, they have a hydrophobic core region and a positively charged N-terminal region, usually with a net charge of +1 or +2. The similarities between signal peptides and TTDs are mainly at the C-terminus of the peptides. The TTDs described here all contain a hydrophobic core and small, uncharged residues at the -1 and -3 positions relative to the TPP processing site. The PC TTD is also similar in length and the net charge of its N-terminus to signal peptides. However, at their N-termini the 23K and 33K TTDs show wide variation when compared with signal peptides. The 23K TTD is around twice as long as an average signal peptide and most of this extra length can be assigned to the N-terminus. This N-terminal portion is highly charged, having a similar net charge to a signal peptide but containing both positively and negatively charged residues, 4 basic and 3 acidic. The 33K TTD is even longer than that of 23K and is also very highly charged, with 4 basic and 4 acidic residues. There are therefore similarities in overall structure between TTDs and signal peptides, but also several differences. TTDs also appear to be more variable among themselves than signal peptides in length and N-terminal charge distribution.

CHAPTER 5 - ANALYSIS OF THE SPP CLEAVAGE SITE OF PRE-33K USING SITE-DIRECTED MUTAGENESIS

5.1 Introduction

As discussed in chapter 4, the presequences of three thylakoid lumen proteins, wheat pre-23K and pre-33K and *Silene pratensis* PC, were all cleaved by SPP between a positively charged residue (arginine or lysine) and an alanine residue. Pre-23K from spinach is also thought to be cleaved between lysine and alanine, within a similar sequence motif to that of wheat pre-23K (E. Wachter, R. G. Herrmann and C. Robinson, unpublished data). This raises the possibility that these residues are important in recognition and cleavage of the presequences by SPP. The residues immediately surrounding the SPP cleavage site have been shown to be important in determining the efficiency of cleavage of pre-LHCP by SPP. Wheat pre-LHCP is a thylakoid membrane protein which is cleaved upon import into the chloroplast at one of two different sites. Cleavage at the primary and secondary sites gives rise to proteins of 26 kDa and 25 kDa respectively (Lamppa and Abad, 1987). SPP is probably responsible for cleavage at the secondary site only (Abad *et al.*, 1989). SPP has been shown to cleave at this site between a lysine and an alanine residue, a site which is similar to the cleavage sites determined for the thylakoid lumen proteins. In wheat, the primary and secondary sites are both recognised efficiently *in vivo*, with a ratio of 26/25 kDa products of 1.5:1. In pea pre-LHCP, however, the ratio 26/25 kDa products is 5:1, suggesting that in this precursor the SPP site is only recognised with a low efficiency. This site contains the residues TT↓KK in the place of the AK↓AK of wheat pre-LHCP, where the arrow denotes the SPP processing site. To determine if this difference accounts for the differences in cleavage efficiency between the two species, the AKA residues of the wheat precursor were mutated to TTK and the TTK of the pea precursor were mutated to AKA, so the secondary cleavage sites in the two species were in effect exchanged (Clark and Lamppa, 1991). This had the effect of

also exchanging the cleavage efficiencies of the proteins, showing that the efficiency of cleavage by SPP is determined in this case entirely by the three residues around the cleavage site. This does not exclude the possibility of the presence of other sites in the precursor which are also responsible for recognition and binding by SPP- these three residues must specify cleavage within a particular sequence context or conformation, as when the AKA motif is moved downstream by four residues, processing is abolished (Clark *et al.*, 1989). Gavel and von Heijne (1990) suggested that a transition from a β-sheet to an α-helix at the cleavage site may be required for processing, but this has yet to be established as an important factor, and does not appear to hold for all precursors.

The importance of the residues around the SPP cleavage site in processing by SPP was therefore investigated in wheat pre-33K. This was approached by using site-directed mutagenesis of the arginine residue N-terminal, and the alanine residue C-terminal, to the cleavage site. The effect of these mutations on cleavage of the precursors by SPP in an organelle-free reaction and on import into isolated chloroplasts was then studied.

5.2 Generation of Mutant Precursor Proteins

The oligodeoxynucleotides used in the mutagenesis reaction were synthesised as described in section 2.17.1 and phenol/chloroform extracted and ethanol precipitated before use. The oligonucleotides were phosphorylated (section 2.17.2), as 5'-phosphate groups are required for completion of the mutant strand synthesis during the mutagenesis reaction. Their sequences are shown below, with the arrow representing the site of cleavage by SPP:

His Val Ala Arg ↓ Ala Phe	
G CAC GTC GCC CGG GCG TTC GG	original
G CAC GTC GCC ATG GCG TTC G	arg → met
G CAC GTC GCC GAG GCG TTC G	arg → glu
G CAC GTC GCC GCG GCG TTC G	arg → ala
C GTC GCC CGG AAG TTC GG	ala → lys

The oligonucleotides were annealed to single-stranded template DNA consisting of the vector M13mp19 containing a 320 bp *Eco*R1/*Kpn*1 fragment encoding the presequence of the wheat 33K precursor protein (Meadows *et al.*, 1991). The mutagenesis reaction was carried out as described (section 2.17.4) and single-stranded DNA isolated from the plaques generated. This DNA was sequenced to ensure that it contained the expected mutation and this sequence data is shown in figure 19. The entire insert was sequenced in each case to ensure that no other mutations had been introduced during the mutagenesis reaction.

The DNA encoding the complete 33K precursor was then reconstructed in M13mp19 (figure 20). The mutant DNA was digested with *Pst*1 to release a 170 bp fragment and the linear vector containing the fragment of the original insert which carried the mutation was isolated. This was ligated with a 1.1 kb fragment generated by digestion of p33K-2 with *Pst*1 to create the entire 33K precursor DNA in M13mp19. Digestion with *Eco*R1 released a 1.3 kb fragment encoding the complete mutant 33K precursor which was subcloned into pGEM-4Z which had previously been linearised with *Eco*R1. This plasmid DNA was sequenced to ensure that the sub-cloning steps were successful and that no further mutations were introduced into the DNA. The plasmid pGEM-4Z contains two promoters, flanking the site at which the required gene is inserted, which promote transcription in opposite directions using two different RNA polymerases, SP6 and T7. The mutants could be ligated into the plasmid in either orientation and so transcribed with one of the RNA polymerases. The mutated pre-33K DNA was transcribed using the appropriate polymerase (section

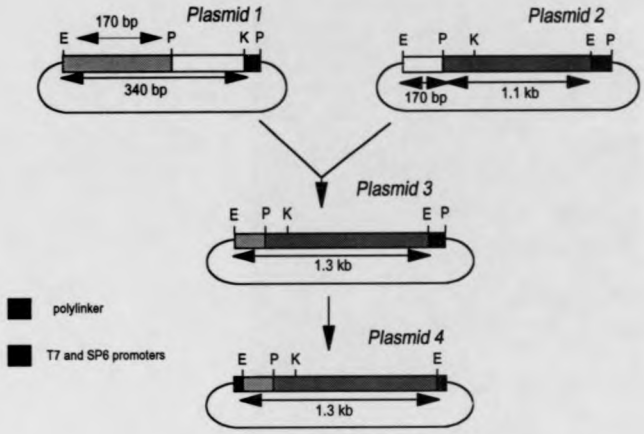
Figure 19 *Site-directed mutagenesis of DNA encoding pre-33K*

The DNA sequence of a portion of template DNA encoding the region around the SPP cleavage site is shown. The corresponding region after site-directed mutagenesis for each of the mutant precursors is also shown.

Arg-Met
A C G T
Arg-Ala
A C G T
Arg-Glu
A C G T
Ala-Lys
A C G T
Original
A C G T

Figure 20 *Strategy for the reconstruction of mutated pre-33K DNAs in pGEM-4Z*

Double-stranded M13mp19 vector containing the mutated 340 base pair fragment of the pre-33K presequence (plasmid 1) was digested with *Pst*1 and the cut vector re-isolated. The p33K-2 plasmid (plasmid 2) was digested with *Pst*1 to release a 1.1 kb fragment which was ligated into plasmid 1 to reconstruct the entire mutant pre-33K DNA (plasmid 3). Plasmid 3 was digested with *Eco*R1 to release a 1.3 kb fragment which was ligated into pGEM-4Z previously linearised with *Eco*R1 to produce the final construct, plasmid 4. (E = *Eco*R1, P = *Pst*1, K = *Kpn*1).


Plasmid 1
170 bp
E
P
K P
340 bp
Plasmid 2
E P K
E P
170 bp
1.1 kb
Plasmid 3
E P K
E P
1.3 kb
Plasmid 4
E P K
E
1.3 kb
polylinker
T7 and SP6 promoters

2.18) and translated in a wheatgerm or rabbit reticulocyte lysate *in vitro* translation system in the presence of [^{35}S]methionine (section 2.19 and 2.20). The translation reactions in each case produced a radiolabelled product of the same size as the non-mutated pre-33K as determined by SDS-PAGE followed by fluorography (figure 21).

5.3 The Mutant Precursor Proteins are Cleaved by SPP at the Correct Site

Incubation of the mutant precursor proteins with SPP revealed that each was cleaved by SPP at a single site, and the cleavage products all appeared to be the same size as that produced by the non-mutated pre-33K (*ie* the intermediate size) as shown by SDS-PAGE. Cleavage took place, however, with varying efficiencies. In particular, the arg → glu and ala → lys mutations greatly reduced cleavage by SPP. Mutagenesis of the TPP cleavage site of the same precursor in some cases caused cleavage at another site, N-terminal to the genuine site, when the usual site was blocked (Shackleton and Robinson, 1991). To ensure that the mutants generated in the present study were being cleaved at the correct site, the intermediate-sized proteins were radiosequenced.

The mutant precursor proteins were synthesised in a rabbit reticulocyte lysate system as described in section 2.20 in the presence of [^{3}H]phenylalanine. The radiolabelled precursors were incubated with an SPP preparation to produce the intermediate forms, which were separated from the precursor proteins by SDS-PAGE. The ala → lys mutant was not processed sufficiently for radiosequencing of the intermediate form, as the amount of intermediate produced was too low. The remaining three intermediate forms were subjected to Edman degradation (performed by B. Dunbar, Aberdeen) and the radioactive counts were determined for each cycle of the degradation (see section 2.27). The pre-33K presequence contains a phenylalanine residue two residues C-terminal to the correct SPP cleavage site, as determined in section 4.3. If the mutant proteins are cleaved at the correct site by SPP, a peak of radioactivity would be expected at cycle 2 of the degradation. This was seen to be the case for all three of the arginine mutants, as shown by the data in

Figure 21 In vitro *transcription and translation of non-mutated and mutant DNAs encoding pre-33K*

cDNAs encoding non-mutated and mutant pre-33K proteins were transcribed *in vitro* as described in section 2.18 and translated in a wheatgerm system in the presence of [^{35}S]methionine (section 2.19). The translation products were analysed by SDS-PAGE and visualised by fluorography (section 2.21).

Original
Arg-Met
Arg-Ala
Arg-Glu
Ala-Lys
42kDa

figure 22. The mutation of the arginine residue immediately N-terminal to the SPP cleavage site therefore does not affect the site of SPP cleavage in the 33K precursor protein.

5.4 Time Course Analysis of the Cleavage of the Mutant Precursor Proteins by SPP

Although the site of SPP cleavage is not affected by the mutations created, at least in the case of the arginine mutations, the efficiency of cleavage is reduced in each of the mutants. This was further investigated by a time course analysis of cleavage. Each mutant protein, and non-mutated pre-33K, was incubated with partially-purified SPP and samples were taken at time points throughout the incubation up to 30 minutes. The samples were analysed by SDS-PAGE and fluorography, and the results shown in figure 23. The intermediate bands were quantified using laser densitometry and rates of cleavage (compared with the non-mutated pre-33K) calculated (figure 24).

It can be seen that the extent of cleavage is reduced in each of the mutants compared with the non-mutated pre-33K, although apparently by very little in the case of the arg → met and arg → ala mutants. As the non-mutated pre-33K and the arg → met and arg → ala mutants were cleaved very rapidly in this experiment, it was not possible to determine true initial rates of cleavage, as early enough time points could not be taken. Dilutions of the SPP preparation used were therefore made and the time course analysis repeated. The results for a five-fold dilution of SPP are shown in figure 24. It can be seen from this that the arg → met and arg → ala mutants are in fact cleaved more slowly than the non-mutated pre-33K, suggesting that the mutated residues are important in the processing reaction. These residues could be directly involved in either binding of the precursor proteins to SPP or in the catalytic mechanism itself. Alternatively, the mutated residues may have a more general effect in altering the conformation of the presequence. It has been proposed that SPP may recognise overall structural features of the presequence rather than specific residues

Figure 22 *Identification of the SPP cleavage site within the presequences of wheat pre-33K mutant proteins arg → met, arg → ala and arg → glu*

Panel A

Sequence of the first 40 residues of the non-mutated pre-33K presequence with an arrow showing the site of cleavage by SPP. The phenylalanine residue labelled for microsequencing analysis of the mutant proteins is underlined.

Panel B

The arg → met mutant 33K precursor protein was synthesised *in vitro* in the presence of [^{3}H]phenylalanine and incubated with an SPP preparation to produce the intermediate-sized form. This cleavage product was subjected to automated Edman degradation and radioactive counts determined for each cycle of the degradation.

Panel C

As panel B but using the arg → glu mutant.

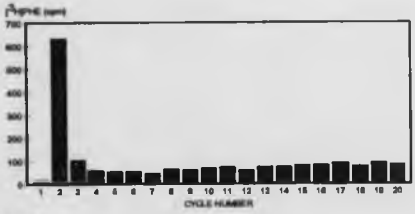
Panel D

As panel B but using the arg → ala mutant.

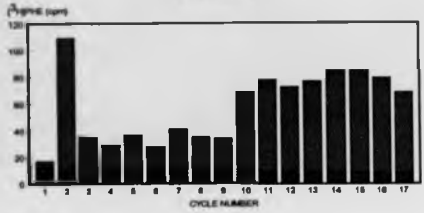
A

MAASLQAAATVMPAKIGGRA
SSARPSSHVARAEGVDAGAR
SPP

B



C



D

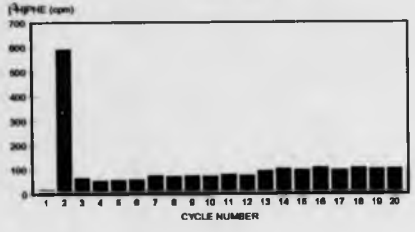


Figure 23 *Time course analysis of the cleavage of non-mutated and mutant pre-33K proteins by SPP in an organelle-free processing reaction*

Non-mutated pre-33K and the arg → met, arg → ala, arg → glu and ala → lys pre-33K mutants were synthesised in a wheatgerm lysate system in the presence of [^{35}S]methionine as described in section 2.19 and incubated with an SPP preparation at 27^{o}C (section 2.26.1). Aliquots were removed from the mixture after 0, 2, 5, 10, 30 and 90 minutes and the reaction stopped by boiling in SDS sample buffer. Samples were analysed by SDS-PAGE and fluorography. Numbers above the tracks indicate the length of time of incubation with SPP at 27^{o}C and the positions of the bands corresponding to the precursor and intermediate forms of 33K are indicated.

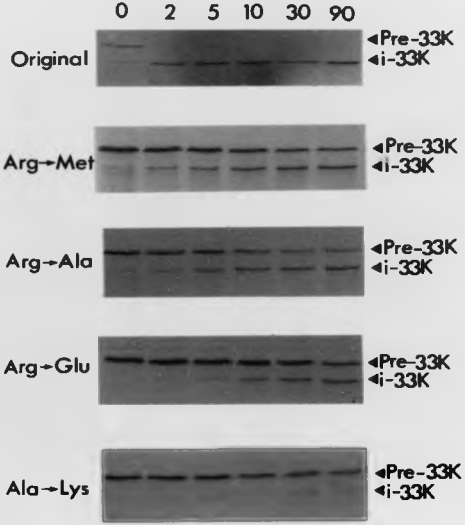

0
2
5
10
30
90
Original
Pre-33K
i-33K
Arg→Met
Pre-33K
i-33K
Arg→Ala
Pre-33K
i-33K
Arg→Glu
Pre-33K
i-33K
Ala→Lys
Pre-33K
i-33K

Figure 24 *Quantification of the time course analysis of the cleavage of non-mutated and mutant 33K precursor proteins by SPP*

Panel A

Precursors were generated by *in vitro* transcription and translation in the presence of [^{35}S]methionine and incubated with an SPP preparation. Samples were taken from the incubation at various time points and analysed by SDS-PAGE and fluorography as described in figure 23. Bands corresponding to the precursor and intermediate sized proteins were quantified by laser densitometry and the percentage of precursor converted to intermediate calculated. This percentage cleavage is plotted against time of incubation.

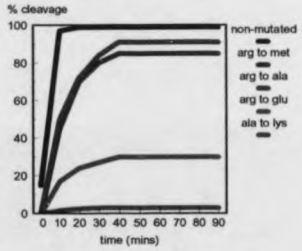
Panel B

As for panel A, but the SPP preparation was diluted 5-fold before incubation with the precursor proteins.

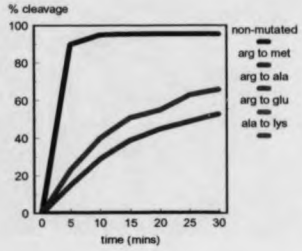
Panel C

As for panel A, but the SPP preparation was diluted 10-fold before incubation with the precursor proteins.

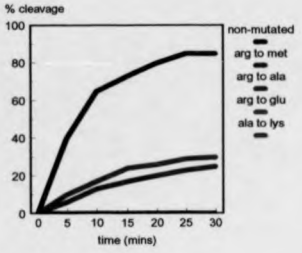
A


% cleavage
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90
time (mins)
non-mutated
arg to met
arg to ala
arg to glu
ala to lys

B


% cleavage
100
80
60
40
20
0
0 5 10 15 20 25 30
time (mins)
non-mutated
arg to met
arg to ala
arg to glu
ala to lys

C

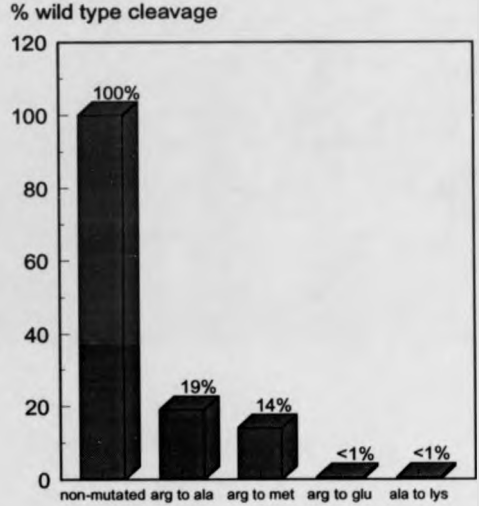

% cleavage
100
80
60
40
20
0
0 5 10 15 20 25 30
time (mins)
non-mutated
arg to met
arg to ala
arg to glu
ala to lys

(Robinson and Ellis, 1985; von Heijne *et al.*, 1989; von Heijne and Nishikawa, 1991) and so alterations in the primary structure of the presequence could cause changes in the secondary structure around the cleavage site. The arginine mutations involved changing the arginine residue adjacent to the cleavage site to methionine, alanine or glutamate. Methionine is of a similar size to arginine but is uncharged, alanine is also uncharged but is much smaller than arginine whereas glutamate is of a similar size to arginine but is negatively charged. The approximate rates of cleavage of each of the mutant precursor proteins compared with the non-mutated pre-33K were determined from the time course analysis of cleavage using different dilutions of the SPP preparation. Figure 25 shows that the introduction of a methionine or alanine residue in the place of arginine reduces the rate of SPP cleavage of pre-33K to around 15-20% of the non-mutated rate. In contrast, the substitution of the arginine residue with a glutamate residue reduces the rate of cleavage to less than 1% of wild-type rate. This suggests that the charge on this residue is more important than the size. Assuming that this residue will be in the active site of SPP when cleavage occurs, it may be envisaged that the positively charged arginine residue binds to some negatively charged residue at the active site of SPP, and that this interaction facilitates the cleavage reaction. This would explain the results obtained, as neutral residues would not be able to bind the proposed acidic residue, resulting in a decreased affinity of the enzyme for the substrate and so an apparent decrease in reaction rate. A negatively charged residue such as glutamate would repel the acidic residue and so the reaction rate would be decreased even further.

The substitution of a lysine residue for the alanine residue immediately C-terminal to the cleavage site has a dramatic effect on cleavage rate, decreasing the reaction rate to less than 1% of wild type rate. This again could be due to an alteration in either charge or size of this residue. Lysine is significantly larger than alanine and so, assuming that this residue also lies within the active site, the lysine residue could prevent the protein from binding efficiently to SPP by being unable to fit into the active site. Alternatively, the positive charge on the lysine residue could prevent

Figure 25 *The rate of cleavage of non-mutated and mutant pre-33K proteins in an organelle-free SPP processing assay*

The rate of cleavage of each mutant precursor compared with non-mutated pre-33K was determined from the gradients of the graphs shown in figure 24.


% wild type cleavage
120
100
80
60
40
20
0
100%
19%
14%
<1%
<1%
non-mutated
arg to ala
arg to met
arg to glu
ala to lys

binding to SPP. This mutation results in two adjacent positively charged residues on either side of the cleavage site which will repel each other and so could make cleavage by SPP unfavourable. To determine the requirements for alanine in this position, further mutant precursor proteins would need to be constructed, both single mutations with the alanine residue replaced by a variety of other residues and double mutations containing substitutions in place of both the arginine and the alanine residues on either side of the SPP cleavage site.

5.5 Import of Mutant Precursor Proteins into Intact Chloroplasts

Incubation of pre-33K with intact pea chloroplasts in the presence of ATP and light results in import of the protein into the thylakoid lumen of the chloroplasts, as determined by fractionation studies and resistance of the imported protein to degradation by externally added protease. Tests were carried out to determine whether the mutations affected binding to envelope membrane receptors prior to import, interaction with import machinery during translocation across the envelope membranes and possible interactions with stromal components required for further targeting to the thylakoid, such as molecular chaperones. After cleavage by SPP, the arginine mutant intermediates are then identical to the non-mutated 33K intermediate and may be expected to follow the usual import route. In the case of the alanine mutant, the intermediate protein now has a lysine residue at its N-terminus, leading to the possibility that this may affect targeting to and translocation across the thylakoid membrane into the thylakoid lumen.

The mutated 33K precursors, synthesised *in vitro* in a wheatgerm lysate system, were incubated with isolated pea chloroplasts as described in section 2.28 to determine the effect of the mutations on the import characteristics of the precursors. The import reactions were treated with thermolysin to digest external protein and thereby demonstrate import into the chloroplasts. Chloroplasts were lysed after import and separated into stromal and thylakoidal fractions to determine the sub-organellar

location of the imported products, and the thylakoids protease-treated to test whether transport into the thylakoid lumen had taken place (Figure 26).

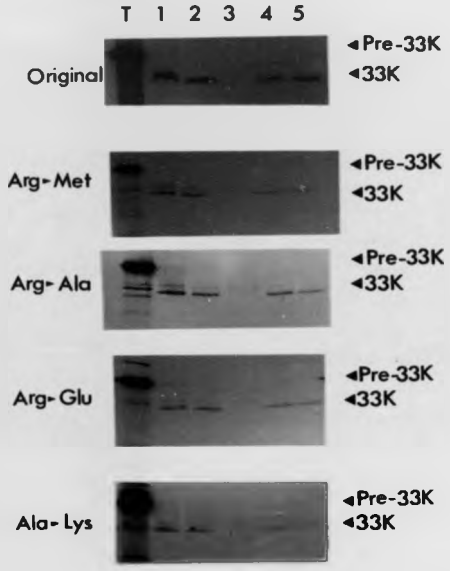
Each of the mutant proteins can be seen to be imported into the thylakoid lumen, as the thermolysin-treated thylakoid tracks all contain labelled protein, indicating that this product is internal to the thylakoids and so is protease-protected. The replacement of alanine by lysine in the ala → lys mutant therefore does not affect intra-organellar targeting of the protein or translocation across the thylakoid membrane. Other events such as envelope receptor binding and translocation are apparently unaffected by any of these mutations. Each mutant is also cleaved by TPP to the mature size, indicating that this processing event is not affected by the mutations. The mutations created do not significantly alter the import characteristics of pre-33K in these respects. This also suggests that the mutations do not alter the conformation of the pre-33K presequence, although it does not rule out the possibility that localised changes in the secondary structure of the presequence have occurred.

5.6 Summary of Mutagenesis Analysis of the SPP Cleavage Site of Pre-33K

The arginine residue immediately N-terminal to the SPP cleavage site of pre-33K was replaced using oligonucleotide-directed mutagenesis by methionine, alanine or glutamate. All three mutant precursor proteins were processed by partially-purified SPP to the correct intermediate-sized product in an organelle-free system. This cleavage reaction occurred at widely varying rates for the three mutants when compared with non-mutated pre-33K cleavage. The arg → met and arg → ala mutants were cleaved at a rate of approximately 15-20% of that of non-mutated pre-33K, whereas the rate of cleavage of the arg → glu mutant was reduced to less than 1% of the original rate. This suggests that the arginine residue in this position plays an important role in cleavage; in particular the positive charge on this residue may be important.

Figure 26 *Import of non-mutated and mutant precursor proteins into isolated chloroplasts*

Precursor proteins were synthesised by *in vitro* translation in a wheatgerm system (section 2.19; lanes T). Isolated intact pea chloroplasts were pre-incubated with 10 mM ATP for 10 minutes at 25°C before addition of the precursor proteins and incubation in the light at 25°C for 20 minutes (section 2.28). Samples were taken after washing with SRM (lanes 1) or after treating with 200 µg/ml thermolysin (lanes 2). Washed chloroplasts were lysed in 10 mM Tris-Cl pH 7.6/5 mM $MgCl_2$ and separated into a stromal fraction (lanes 3) and a thylakoidal fraction. The thylakoidal fraction was analysed after washing in 10 mM Tris-Cl pH 7.6/5 mM $MgCl_2$ (lanes 4) or after washing followed by treatment with 200 µg/ml thermolysin (lanes 5). All samples were analysed by SDS-PAGE and fluorography.


T 1 2 3 4 5
Original
◂Pre-33K
◂33K
Arg-Met
◂Pre-33K
◂33K
Arg-Ala
◂Pre-33K
◂33K
Arg-Glu
◂Pre-33K
◂33K
Ala-Lys
◂Pre-33K
◂33K

The alanine residue immediately C-terminal to the SPP cleavage site was replaced by a lysine residue using site-specific mutagenesis and found to be cleaved by SPP very inefficiently in an organelle-free reaction. The rate of cleavage was greatly reduced compared with the rate of cleavage of non-mutated pre-33K, to less than 1% of the original rate. Alanine is a small amino acid and so the larger lysine residue may be unable to fit into the active site of SPP and so reduce the cleavage rate in this way. Alternatively, the charge on the lysine residue may disrupt the binding of the ala → lys mutant to SPP, or the catalytic mechanism of the reaction.

All four of the mutant precursor proteins described are imported into intact isolated chloroplasts, translocated into the thylakoid lumen and processed to the mature size with no detectable differences between the mutants and non-mutated pre-33K. As it is not known whether the full 33K precursor protein can cross the thylakoid membrane, or only the intermediate form, the extent of processing by SPP within the organelle cannot be determined. The uncleaved precursor may be translocated into the thylakoid lumen and cleaved to the mature size or alternatively the SPP cleavage efficiency may be greater in the chloroplast than in an organelle-free system due to the precursor being in an unfolded conformation, or to a higher effective concentration of SPP.

CHAPTER 6 - CLEAVAGE OF MITOCHONDRIAL PRECURSOR PROTEINS BY SPP

6.1 Introduction

Plant cells contain both chloroplasts and mitochondria and so must contain highly specific targeting signals to direct newly-synthesised proteins to the correct organelle. It has been suggested that the stromal targeting signals of higher plants tend to have an overall positive charge, to be rich in serine and threonine residues and to contain an amphiphilic β-strand immediately before the SPP cleavage site (von Heijne *et al.*, 1989). Mitochondrial targeting signals also contain a high proportion of positively-charged and hydroxylated residues but, unlike chloroplast targeting signals, the central regions have the potential to form amphiphilic α-helices which can interact with lipid bilayers (Roise *et al.*, 1986; Roise *et al.*, 1988; von Heijne *et al.*, 1989). An exception is the presequence of the mitochondrial protein cytochrome oxidase subunit Va which will target a reporter protein to both mitochondria and chloroplasts (Huang *et al.*, 1990). This presequence is unlike a typical mitochondrial targeting sequence, containing no amphiphilic α-helix, and the import characteristics of this protein are also unusual. However, the specificity of targeting of several proteins to the chloroplast or mitochondrion has been shown by Boutry *et al.* (1987), de Boer *et al.* (1988) and Whelan *et al.* (1990), suggesting that in general targeting is highly specific.

Due to the high specificity of targeting signals, it has previously been considered that the processing enzymes of mitochondria and chloroplasts would be equally specific. Attempts have been made to process various non-chloroplast proteins with SPP, such as rotavirus mRNA translation products (Robinson and Ellis, 1984a) and pre-ricin B chain (provided by Dr R. Wales, Warwick; data not shown) and no cleavage products have been seen. However, cleavage of mitochondrial protein precursors by a stromal extract was reported by Whelan *et al.* (1991),

although chloroplast protein precursors were not cleaved by a mitochondrial matrix extract. This work was carried out using crude stromal and mitochondrial matrix preparations, and stromal extracts have been shown to contain a large number of protein species, including several endopeptidases (Musgrove *et al.*, 1989) and aminopeptidases (Liu and Jagendorf, 1986). Protein presequences are thought to have an unfolded conformation (von Heijne and Nishikawa, 1991) and so it is possible that general proteases in the stromal extract may digest mitochondrial presequences, leaving the mature protein intact. In addition, the stromal extract used by Whelan *et al.* did not cleave chloroplast precursor proteins, suggesting that either the SPP in the stromal extract was inactive, in which case it could not be responsible for cleavage of the mitochondrial precursor proteins and so cleavage must be due to another protease in the stromal extract, or that the precursor used was in a conformation which did not allow cleavage, thus invalidating the results obtained for the non-cleavage of the chloroplast precursors by the mitochondrial extract. Observations by A. Creighton (MSc thesis) suggested that three mitochondrial precursor proteins, the precursors of MPP, the β subunit of the F_1 ATPase ($F_1\beta$) and the non-haem iron sulphur protein of the ubiquinol cytochrome *c* oxidoreductase complex (Fe/S), were cleaved by a partially purified pea SPP preparation. These observations were therefore investigated further.

6.2 Cleavage of Mitochondrial Precursor Proteins

Mitochondrial precursor proteins which are cleaved efficiently by MPP/PEP *in vitro* in an organelle-free system were chosen for further work on the processing specificities of SPP and MPP. cDNA clones encoding the mitochondrial protein precursors chosen, MPP (Hawlitschek *et al.*, 1988), $F_1\beta$ (Rassow *et al.*, 1990), Fe/S (Harnisch *et al.*, 1985) and cyclophilin (Tropschug *et al.*, 1988) from *Neurospora crassa* and a yeast truncated cytochrome b_2 - DHFR fusion protein ($b_2\Delta19$; Arretz *et al.*, 1991), were obtained from Prof. W. Neupert (Munich). The clones were transcribed using SP6 RNA polymerase (section 2.18) and the mRNAs produced

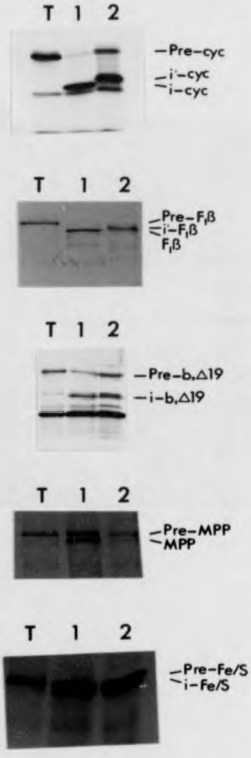
were translated in a rabbit reticulocyte lysate system (section 2.20) to produce [^{35}S]methionine-labelled proteins of the expected sizes. These precursors were incubated with partially-purified pea SPP or with MPP and PEP from *Neurospora crassa* as described in section 2.26 and the products of the reaction visualised using SDS-PAGE followed by fluorography.

Figure 27 shows the results of this analysis. All of the mitochondrial proteins were cleaved by the partially-purified SPP extract as well as by the MPP/PEP. The MPP and Fe/S precursors were cleaved by the SPP-containing extract to a product of approximately the same size as that generated by MPP/PEP cleavage, as expected from the previous work described. The $b_2\Delta19$ precursor was also cleaved to approximately the same size by both MPP and the SPP-containing extract. The $F_1\beta$ and cyclophilin precursors, however, were cleaved by the SPP extract to a size clearly different to that of the product of MPP/PEP cleavage. In the case of $F_1\beta$, this is contrary to previous reports stating that both extracts cleave $F_1\beta$ to the same size (Whelan *et al.*, 1991; A. Creighton, MSc Thesis) which may be due to different gel systems used. $F_1\beta$ appears to be cleaved by the SPP preparation at more than one site, as close inspection reveals three minor bands in addition to the major band produced. This suggests that, at least for $F_1\beta$ and cyclophilin, the partially-purified extract containing the SPP activity does not cleave the mitochondrial precursors at the correct site (the site recognised by MPP) but that the enzyme responsible for cleavage is recognising a different site within the presequence of these proteins. It is also possible that the other mitochondrial precursors tested are cleaved at different sites by MPP and the SPP extract, but that the gel system does not resolve the two forms.

The chloroplast precursor proteins pre-23K, pre-33K and pre-PC were also incubated with MPP/PEP or partially-purified SPP to determine whether the mitochondrial enzyme would cleave precursors normally targeted to the thylakoid lumen. No lower molecular weight products were seen on incubation with MPP/PEP, showing that MPP is specific for mitochondrial precursor proteins and will not cleave

Figure 27 *Processing of mitochondrial precursor proteins by* Neurospora crassa *MPP and a pea SPP preparation*

Precursors to five mitochondrial precursor proteins, cyclophilin, $F_1\beta$, $b_2\Delta 19$, MPP and Fe/S, were synthesised in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine (section 2.20). The translation products (lane T) were incubated with MPP/PEP for 45 minutes at 30°C (lane 1) and a pea SPP preparation for 90 minutes at 27°C (lane 2) as described in section 2.26, and analysed by SDS-PAGE followed by fluorography.


T 1 2
Pre-cyc
i'-cyc
i-cyc
T 1 2
Pre-F1β
i-F1β
F1β
T 1 2
Pre-b,Δ19
i-b,Δ19
T 1 2
Pre-MPP
MPP
T 1 2
Pre-Fe/S
i-Fe/S

chloroplast proteins. The SPP extract processed all three proteins to the intermediate size as expected (see section 4; data not shown).

6.3 The Partially-Purified SPP Extract Does Not Contain MPP Activity

The first possibility considered for the mitochondrial protein processing activity present in the partially-purified SPP sample was that the sample contained a small amount of pea MPP and PEP due to contamination of the chloroplast preparation with mitochondria. The centrifugation step in chloroplast preparation (section 2.22.2) should not result in the pelleting of the mitochondria in the total extract but some contamination is possible. Although the SPP preparation and MPP cleaved some of the mitochondrial precursor proteins to different sizes, the MPP and PEP used were from *Neurospora crassa* mitochondria and it is possible that higher plant MPP will cleave *Neurospora* protein precursors at a different site to *Neurospora* MPP. Pea MPP could therefore be responsible for the cleavage activity present.

6.3.1 Detection of a Mitochondrial Protein by Western Blotting

To address this problem, antibodies raised against *Neurospora crassa* mitochondrial $F_1\beta$ (obtained from Prof. W. Neupert, Munich) were used to detect the $F_1\beta$ protein in maize mitochondrial extract (section 2.25; mitochondria kindly provided by A. Liddell, Oxford), total pea cell extract (made by filtering a pea leaf homogenate through eight layers of muslin), crude chloroplasts (section 2.22.2) and Percoll-purified chloroplasts (section 2.28.1). Western blotting (section 2.30) was performed using the $F_1\beta$ antibodies and bands were visualised using ECL. The results are shown in figure 28.

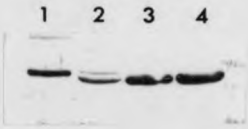
In the track containing the maize mitochondrial extract, a single major band is seen at around 55 kDa, approximately the expected size for a higher plant mitochondrial $F_1\beta$ (Hamasur and Glaser, 1992; Hamasur *et al.*, 1992). The antibodies raised against the *Neurospora* protein therefore recognise the maize protein. In the total pea cell extract track, two bands are seen at approximately the expected size.

Figure 28 ***Western blot of mitochondrial and chloroplast preparations using mitochondrial F$_1\beta$ antibodies***

Proteins in either 10 µl of maize mitochondrial extract, prepared as described in section 2.25 (lane 1), a total pea leaf extract, prepared by filtering a pea leaf homogenate through eight layers of muslin (section 2.22.2; lane 2), a crude chloroplast preparation (section 2.22.2; lane 3) or a Percoll-purified chloroplast preparation (section 2.28.1; lane 4) (with each pea extract containing 3 µg chlorophyll) were analysed by SDS-PAGE followed by transfer onto a nitrocellulose membrane (section 2.30). Proteins cross-reacting with mitochondrial F1β antibodies were visualised by ECL and exposure to X-ray film.

Figure 28 ***Western blot of mitochondrial and chloroplast preparations using mitochondrial $F_1\beta$ antibodies***

Proteins in either 10 µl of maize mitochondrial extract, prepared as described in section 2.25 (lane 1), a total pea leaf extract, prepared by filtering a pea leaf homogenate through eight layers of muslin (section 2.22.2; lane 2), a crude chloroplast preparation (section 2.22.2; lane 3) or a Percoll-purified chloroplast preparation (section 2.28.1; lane 4) (with each pea extract containing 3 µg chlorophyll) were analysed by SDS-PAGE followed by transfer onto a nitrocellulose membrane (section 2.30). Proteins cross-reacting with mitochondrial F1β antibodies were visualised by ECL and exposure to X-ray film.


1
2
3
4

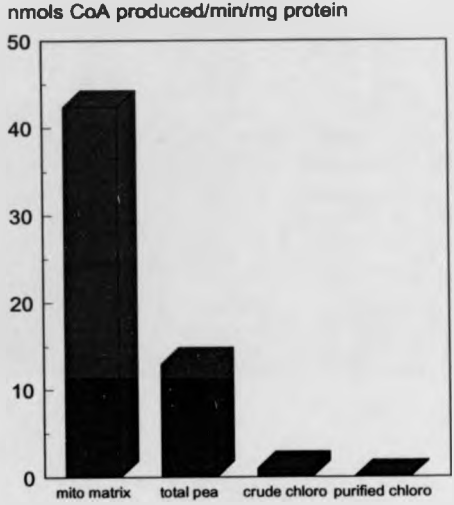
One runs at the same mobility as the maize $F_1\beta$ and so is deduced to be the pea mitochondrial $F_1\beta$. The other band appears from the gel to be slightly smaller than the mitochondrial $F_1\beta$ and is probably the thylakoidal $CF_1\beta$ (Frasch *et al.*, 1989; Kasamo *et al.*, 1989) which the antibodies against the mitochondrial protein may recognise. The total cell extract therefore contains both mitochondrial $F_1\beta$ and thylakoidal $CF_1\beta$. The crude chloroplast and Percoll-purified chloroplast tracks, in contrast, contain only the smaller protein, with no detectable mitochondrial F1β present. This suggests that the method used for isolating chloroplasts does not result in detectable levels of contamination by mitochondria.

6.3.2 Citrate Synthase Assays

Further evidence for the absence of mitochondrial contamination in chloroplast preparations was obtained by assaying the three pea extracts (total extract, crude chloroplasts and Percoll-purified chloroplasts) and a control maize mitochondrial extract for citrate synthase activity, a tricarboxylic acid cycle enzyme found in mitochondria (see section 2.31). In each case, the amount of protein in the sample was determined using the Bio-Rad protein assay after acetone precipitation of the protein to remove chlorophyll, and enzyme activity per mg of protein was calculated. The mitochondrial extract contained high levels of citrate synthase activity as expected. The total pea extract contained a lower level of activity (about 30% that of the mitochondrial level) whereas the two chloroplast preparations contained no detectable citrate synthase activity (figure 29). The chloroplast preparations used for the extraction of SPP activity therefore did not contain detectable levels of two relatively abundant mitochondrial proteins.

Figure 29 *Assay for citrate synthase activity in mitochondrial and chloroplast preparations*

Samples as described in figure 28 were assayed for citrate synthase activity as described in section 2.31, and the amount of CoA produced per minute per mg of protein was plotted for each sample.


nmols CoA produced/min/mg protein
50
40
30
20
10
0
mito matrix
total pea
crude chloro
purified chloro

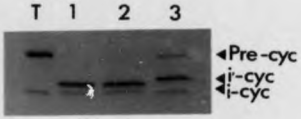
6.4 *Cyclophilin Precursor is Cleaved to the Same Size by Maize and* N. crassa *MPP*

To confirm these results, the processing activities of *Neurospora crassa* purified MPP and maize mitochondrial extract were compared. If *Neurospora* and maize MPP cleave at the same site, then it is probable that pea MPP will also cleave at this site. *In vitro* synthesised *Neurospora* cyclophilin precursor protein was chosen as a substrate as this shows the largest size difference between the products formed by incubation with partially-purified SPP and MPP/PEP. Radiolabelled cyclophilin precursor was incubated with *Neurospora* MPP/PEP, maize mitochondrial extract, or partially-purified pea SPP and the reaction products separated by SDS-PAGE and visualised by fluorography (Figure 30).

The results show that maize MPP and *Neurospora* MPP cleave pre-cyclophilin to the same size, but to a different size to the product of cleavage by the SPP preparation. This strongly suggests that there is no detectable MPP activity in the SPP preparation as no product can be seen corresponding to the mature size protein formed by incubation with either *Neurospora* or maize MPP. The processing activity seen must therefore be due to another protease activity present in the extract, either SPP or a general stromal protease. The stroma contains a variety of peptidases including aminopeptidases (Liu and Jagendorf, 1986) and endopeptidases (Musgrove *et al.*, 1989). However, even the most highly purified SPP preparations will cleave the cyclophilin precursor and these contain no detectable endoprotease activity (other than SPP) when incubated with chloroplast precursor proteins.

Figure 30 *Cleavage of pre-cyclophilin by* Neurospora crassa *MPP, a maize mitochondrial extract and a pea SPP preparation*

Pre-cyclophilin was synthesised by *in vitro* transcription (section 2.18) and translation in a rabbit reticulocyte lysate system (section 2.20). The translation product (lane T) was incubated with *N. crassa* MPP/PEP for 45 minutes at 30°C (lane 1), maize mitochondrial extract for 90 minutes at 30°C (lane 2) or a pea SPP preparation for 90 minutes at 27°C (lane 3) (see section 2.26). The products of the reaction were analysed by SDS-PAGE followed by fluorography.


T
1
2
3
Pre-cyc
i'-cyc
i-cyc

6.5 Elution of Processing Activity from a Gel Filtration Column

The enzyme in the SPP preparation responsible for the cleavage of mitochondrial precursor proteins was then investigated. Two possibilities were considered, that cleavage was catalysed either by SPP itself or by a contaminating general protease in the preparation. The first approach taken was to determine whether the protease activity cleaving mitochondrial precursor proteins could be separated from the SPP activity by column chromatography. A preparation of pea stroma isolated from Percoll-purified chloroplasts was chromatographed on a Sephacryl S-300 gel filtration column (section 2.23.2) and 3 ml fractions collected. Fractions were incubated with either radiolabelled wheat pre-33K or *Neurospora* pre-cyclophilin as described in section 2.26. The reaction products were analysed by SDS-PAGE and fluorography and the results are shown in figure 31.

The percentage of precursor protein cleaved to a smaller size (the intermediate size in the case of pre-33K) was determined by laser densitometry of the exposed film. Figure 32 shows that the peaks of activity for cleavage of pre-33K or pre-cyclophilin by column fractions coincide precisely. The cleavage activities therefore elute from the column in the same elution volume. This shows that the enzymes responsible for the cleavage activities have the same hydrodynamic volumes and so suggests that the same enzyme or two closely related enzymes may in fact cleave both proteins.

6.6 Elution of Processing Activity from an Ion Exchange Column

The above experiment was repeated, with the stromal proteins being separated using a different property. A pea stromal preparation was loaded onto a Q-Sepharose anion exchange column (section 2.23.3) and eluted using a 100 ml gradient of 0 - 500 mM NaCl. 4 ml fractions were collected and fractions were incubated with radiolabelled wheat pre-33K or *Neurospora crassa* pre-cyclophilin synthesised *in vitro* in a rabbit reticulocyte lysate translation system (section 2.20; section 2.26). The reaction products were analysed by SDS-PAGE and fluorography (figure 33).

Figure 31 *Cleavage of pre-33K and pre-cyclophilin by fractions eluted from a Sephacryl S-300 gel filtration column*

Panel A

[^{35}S]methionine-labelled pre-33K was synthesised by *in vitro* transcription and translation. 20 µl of every third fraction eluted from a Sephacryl S-300 gel filtration column was incubated with 1 µl of the translation mixture for 90 minutes at 27^{o}C followed by SDS-PAGE and fluorography. Numbers above the lanes indicate fraction numbers. The sizes of pre-33K and int-33K are labelled.

Panel B

As for panel A but with pre-cyclophilin as the substrate.

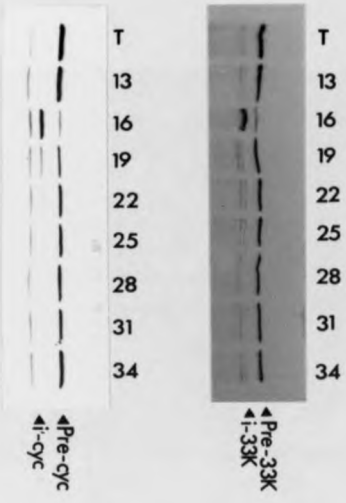

T
13
16
19
22
25
28
31
34
Pre-cyc
i-cyc
T
13
16
19
22
25
28
31
34
Pre-33K
i-33K

Figure 32 *Co-elution of activities cleaving pre-33K and pre-cyclophilin from a Sephacryl S-300 gel filtration column*

Bands from the experiment shown in figure 31 were quantified by laser densitometry and the percentage of cleavage by the SPP preparation calculated. This is plotted against the fraction number from the gel filtration column. The amount of protein present in each fraction is also plotted as the optical density at 280 nm of each fraction.

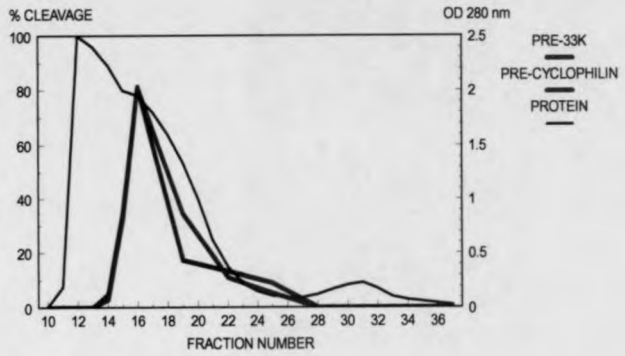

% CLEAVAGE
OD 280 nm
100
80
60
40
20
0
2.5
2
1.5
1
0.5
0
10 12 14 16 18 20 22 24 26 28 30 32 34 36
FRACTION NUMBER
PRE-33K
PRE-CYCLOPHILIN
PROTEIN

Figure 33 *Cleavage of pre-33K and pre-cyclophilin by fractions eluted from a Q-Sepharose anion exchange column*

Panel A

[^{35}S]methionine-labelled pre-33K was synthesised by *in vitro* transcription and translation. 20 µl of every third fraction eluted from a Q-Sepharose anion exchange column was incubated with 1 µl of the translation mixture for 90 minutes at 27°C followed by SDS-PAGE and fluorography. Numbers above the lanes indicate fraction numbers. The sizes of pre-33K and int-33K are labelled.

Panel B

As for panel A but with pre-cyclophilin as the substrate.

Pre-cyc
i-cyc
T
1
3
5
7
9
11
13
15
17
19
21
23
Pre-33K
i-33K
T
1
3
5
7
9
11
13
15
17
19
21
23

The percentage cleavage of the precursors was determined by laser densitometry of the exposed film (figure 34). The peaks of activity cleaving pre-33K and pre-cyclophilin can be seen to coincide, showing that the enzymes cleaving the two proteins elute at the same NaCl concentration.

As the two activities co-elute from both a gel filtration column and an ion exchange column, this provides strong evidence that the same protein is responsible for the cleavage of both chloroplast and mitochondrial precursor proteins, although this is not definitive proof and it is possible that the chloroplast extract could contain two proteins with very closely related properties.

6.7 Effect of Inhibitors on Processing Activity

To obtain more information on the identity of the processing activity in the SPP preparation which cleaves pre-cyclophilin, the effect of various protease inhibitors on processing of pre-cyclophilin and pre-33K was studied. Pea SPP is known to be inhibited by divalent metal ion-chelating agents such as EDTA and 1,10-phenanthroline (Robinson and Ellis, 1984). No other protease inhibitors tested had any inhibitory effect on processing activity. The assays described for processing of chloroplast and mitochondrial precursor proteins contain the serine protease inhibitor PMSF to inhibit any general proteases in the reaction mixtures, and this was found to have no effect on the efficiency of the SPP preparation in processing any of the precursor proteins.

Partially-purified SPP samples (50 µl) were incubated on ice for 20 minutes with one of the following protease inhibitors:

(a) 5 mM 1,10-phenanthroline, a divalent cation chelator,

(b) 5 mM NEM, a cysteine protease inhibitor,

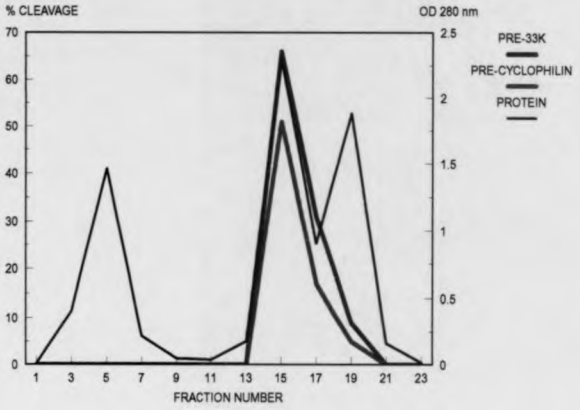
(c) 5 mM E-64, a cysteine protease inhibitor,

(d) 10 mM iodoacetic acid (IAA), a cysteine protease inhibitor.

As some of the inhibitors could also modify the precursor proteins in an assay, the samples containing NEM or IAA were then dialysed overnight against 20 mM

Figure 34 *Co-elution of activities cleaving pre-33K and pre-cyclophilin from a Q-Sepharose anion exchange column*

Bands from the experiment shown in figure 33 were quantified by laser densitometry and the percentage of cleavage by the SPP preparation calculated. This is plotted against the fraction number from the ion exchange column. The amount of protein present in each fraction is also plotted as the optical density at 280 nm of each fraction.


% CLEAVAGE
OD 280 nm
70
60
50
40
30
20
10
0
2.5
2
1.5
1
0.5
0
1
3
5
7
9
11
13
15
17
19
21
23
FRACTION NUMBER
PRE-33K
PRE-CYCLOPHILIN
PROTEIN

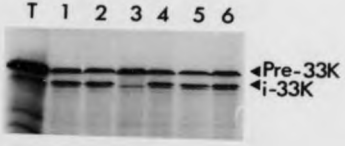
Tris-Cl pH 8.0 to remove any excess inhibitor. As NEM and 1,10-phenanthroline were added from stock solutions made up in ethanol, an equivalent amount of ethanol was added to control reactions to ensure that the ethanol had no effect on SPP activity. The samples were assayed for cleavage of pre-33K and pre-cyclophilin and the results analysed by SDS-PAGE and fluorography (figure 35).

The results show that the processing activity in the SPP preparation which cleaves pre-cyclophilin is inhibited almost completely by 1,10-phenanthroline, but not significantly inhibited by IAA, E-64 or NEM at the concentrations used. These results are almost identical to the effects of the inhibitors on the cleavage of pre-33K. The susceptibility to protease inhibitors of the activities therefore appears to be the same.

SPP is also inhibited by EDTA, with a concentration of about 5 mM or higher required for significant inhibition (Robinson and Ellis, 1984; C. Robinson, unpublished data). An SPP preparation was incubated with various concentrations of EDTA ranging from 0 mM to 5 mM for 20 minutes on ice and then assayed for cleavage of pre-cyclophilin. Figure 36 shows that cleavage of pre-cyclophilin is also inhibited by EDTA, with a concentration of 5 mM required for a significant effect. Lower concentrations of EDTA only cause a small reduction in the extent of processing seen. This again provides evidence that the same enzyme in the SPP preparation is responsible for the processing of both chloroplast and mitochondrial precursor proteins, SPP itself. It will therefore be assumed from this point that the enzyme cleaving the mitochondrial precursor proteins is SPP.

Figure 35 *Effect of inhibitors on the cleavage of pre-cyclophilin and pre-33K by an SPP preparation*

Radiolabelled precursor proteins (lane T) were synthesised by *in vitro* translation in a rabbit reticulocyte lysate system. Precursors were incubated with 50 µl of an SPP preparation (lanes 1), SPP preparation dialysed overnight against 20 mM Tris-Cl pH 8.0 (lanes 6) or SPP preparation which had been pre-incubated for 20 minutes on ice with iodoacetic acid (10 mM; lanes 2), 1,10-phenanthroline (5 mM; lanes 3), E-64 (5 mM; lanes 4) or N-ethylmaleimide (5 mM; lanes 5). After incubation with iodoacetic acid or N-ethylmaleimide, the SPP preparation was dialysed overnight against 20 mM Tris-Cl pH 8.0 to remove any excess inhibitor before incubation with the precursors. Reaction products were analysed by SDS-PAGE and fluorography.


T 1 2 3 4 5 6
Pre-33K
i-33K

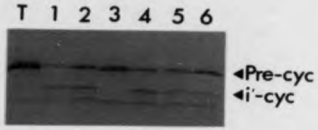
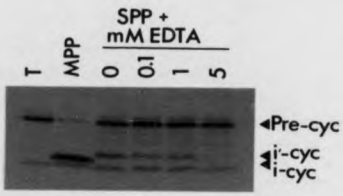

T 1 2 3 4 5 6
Pre-cyc
i-cyc

Figure 36 *Effect of EDTA on the cleavage of pre-cyclophilin by an SPP preparation*

Pre-cyclophilin was synthesised in a rabbit reticulocyte lysate system (section 2.20) and the translation product (lane T) was incubated with MPP/PEP for 45 minutes at 30°C (lane MPP) or with an SPP preparation in the presence of 0, 0.1, 1 or 5 mM EDTA for 90 minutes at 27°C. The products were analysed by SDS-PAGE and fluorography.


SPP +
mM EDTA
T
MPP
0
0.1
1
5
Pre-cyc
i'-cyc
i-cyc

6.8 Requirements for the Activity of MPP

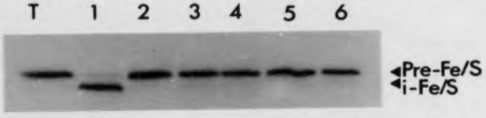
The *Neurospora crassa* processing enzyme which removes the presequences from proteins imported into the mitochondrial matrix consists of two components, MPP and PEP (Hawlitschek *et al.*, 1988). Initially, it was reported that MPP alone had a low processing activity and that PEP was required to stimulate this activity. Recently, however, studies using *E. coli* expressed precursor proteins have shown that MPP in fact has no activity in the absence of PEP and so there is an absolute requirement for both proteins in order for processing to occur (Dr M. Arretz and Prof W. Neupert, pers. comm.). Initial experiments had been performed using radiolabelled precursor proteins synthesised in a rabbit reticulocyte lysate system, and the reticulocyte lysate was found to contain enough PEP to allow a small amount of cleavage in the absence of added PEP, so explaining the earlier reports.

The possibility was then addressed that the SPP sample may contain similar components and so may be able to substitute for one of the components of the reaction. The SPP preparations used have only a very low processing activity when incubated with pre-Fe/S from *Neurospora* mitochondria, whereas MPP/PEP will cleave this precursor efficiently. SPP was incubated with pre-Fe/S in the presence of either MPP or PEP to determine whether SPP could take the place of either of these proteins in the processing reaction. The reaction products were analysed by SDS-PAGE followed by fluorography (figure 37).

It can be seen that the only combination of proteins giving efficient cleavage of the precursor is when MPP and PEP are both present. No stimulation of activity is observed when MPP or PEP alone is present with SPP. SPP cannot therefore substitute for MPP or PEP in the cleavage of mitochondrial precursor proteins.

Figure 37 *Incubation of pre-Fe/S with different combinations of MPP, PEP and SPP*

Pre-Fe/S was synthesised in a rabbit reticulocyte lysate *in vitro* translation system in the presence of [^{35}S]methionine (lane T) and incubated with MPP and PEP (lane 1), MPP alone (lane 2), MPP and SPP (lane 3), PEP and SPP (lane 4), PEP alone (lane 5) or SPP alone (lane 6) for 45 minutes at 30^{0}C. Samples were analysed by SDS-PAGE and fluorography.


T
1
2
3
4
5
6
Pre-Fe/S
i-Fe/S

6.9 *Determination of the MPP Cleavage Site in the* N. crassa *Cyclophilin Presequence*

The next aim was to determine the site at which SPP cleaves within the pre-cyclophilin presequence to enable a comparison to be made between the primary structures around the cleavage sites of chloroplast and mitochondrial precursor proteins, and between the cleavage sites of MPP and SPP within the same precursor. However, the MPP cleavage site of this precursor was not known unequivocally. Pre-cyclophilin is cleaved by MPP on import into mitochondria to an intermediate size, and this intermediate is cleaved to the mature size by another matrix-located protease, the mitochondrial intermediate peptidase (MIP; Tropschug *et al.*, 1988; Kalousek *et al.*, 1988). The MIP cleavage site was determined by protein sequencing of the amino-terminus of the mature protein and the MPP cleavage site deduced to be eight amino acid residues N-terminal to this (Isaya *et al.*, 1991), but the MPP cleavage site was not determined directly. It was therefore decided to confirm the position of the MPP cleavage site before attempting to compare this with cleavage by the SPP sample. The approach used was essentially as described in section 4, with the Edman degradations carried out by B. Dunbar (Aberdeen).

Pre-cyclophilin was synthesised in a rabbit reticulocyte lysate *in vitro* translation system in the presence of [^{3}H]phenylalanine to produce radiolabelled protein. This was processed to the intermediate size by incubation with MPP and PEP and the intermediate separated from the precursor form by SDS-PAGE. The intermediate form was subjected to automated Edman degradation and counts determined for each cycle of the degradation, shown in figure 38.

A large peak can be seen at cycle 1 of the degradation, indicating that MPP cleaves before a phenylalanine residue. Two small peaks were also found at cycles 12 and 13 of the degradation. A comparison with the protein sequence (Tropschug *et al.*, 1988) shows that to produce this pattern of peaks of radioactivity, MPP must cleave at the expected position in the presequence, eight residues before the terminal

Figure 38 *Identification of the MPP cleavage site within the* Neurospora crassa *pre-cyclophilin presequence*

Panel A

Sequence of the first 50 amino acid residues of pre-cyclophilin. Arrows denote the MIP and deduced MPP cleavage sites. The residue radiolabelled for the Edman degradation analysis is underlined.

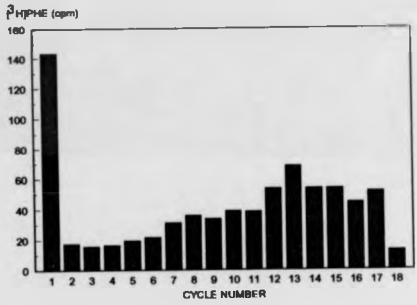
Panel B

[^{3}H]phenylalanine-labelled pre-cyclophilin was generated by *in vitro* transcription and translation and incubated with MPP and PEP to produce the intermediate form. This intermediate was subjected to Edman degradation and counts determined for each cycle of the degradation.

A

MFGPRHFSVLKTTGSLVSSTFSSSL
KPTATFSCARAFSQTSSIMSKVFFD
MPP MIP

B



cleavage site. This also confirms that MIP removes an octapeptide from the intermediate size protein to form the mature protein.

6.10 Determination of the Site of Cleavage of N. crassa *Pre-Cyclophilin by a Partially-Purified SPP Preparation*

The site of cleavage by an SPP preparation within the cyclophilin presequence was investigated using the same technique. Pre-cyclophilin was synthesised in a rabbit reticulocyte lysate *in vitro* translation system in the presence of [^{35}S]methionine to produce radiolabelled protein. This protein was incubated with partially-purified SPP and the reaction products were separated by SDS-PAGE. The cleavage product was subjected to automated Edman degradation and each fraction counted for ^{35}S radioactivity. The results of the degradation are shown in figure 39B. A small peak is found at cycle 13 of the degradation, although the background is high. There is only one methionine residue near the N-terminus of the protein (excluding the initiator methionine) and this is at position -1 in the presequence (*ie* immediately N-terminal to the final MIP cleavage site). For a single peak to be found at this position, SPP must cleave the precursor between a phenylalanine and a serine residue, 5 residues N-terminal to the MPP cleavage site.

As only a small peak was found compared with background levels when the protein was labelled with [^{35}S]methionine, the procedure was repeated using [^{3}H]phenylalanine to label the pre-cyclophilin. As there are several phenylalanine residues in the cyclophilin presequence, the pattern of release of radioactive residues should enable confirmation of the cleavage site. The counts obtained from the Edman degradation of the processed form after incubation with the SPP preparation are shown in figure 39C.

The pattern of peaks of radioactivity confirms the result obtained from the [^{35}S]methionine-labelled protein. The SPP preparation cleaves between phenylalanine and serine to produce peaks of radiolabelled phenylalanine at cycles 6,17 and 18.

Figure 39 ***Identification of the SPP cleavage site within the*** Neurospora crassa ***pre-cyclophilin presequence***

Panel A

Sequence of the first 50 residues of pre-cyclophilin. Arrows denote the sites of cleavage by SPP, MPP and MIP. Residues radiolabelled for the Edman degradation analysis are underlined.

Panel B

[^{35}S]methionine-labelled pre-cyclophilin was generated by *in vitro* transcription and translation and incubated with SPP to produce a lower molecular weight form. This cleavage product was subjected to Edman degradation and counts determined for each cycle.

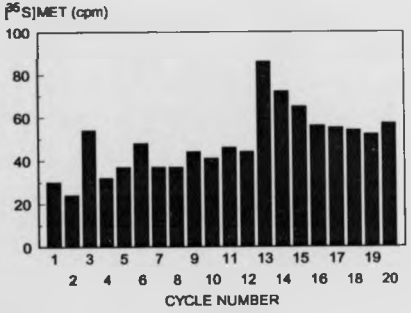
Panel C

As panel B but pre-cyclophilin was labelled with [^{3}H]phenylalanine.

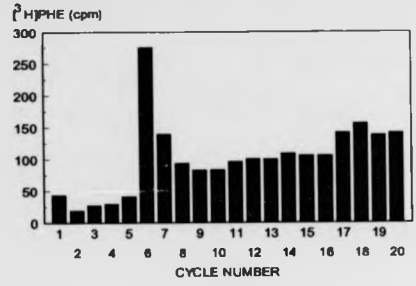
A

MFGPRHFSVLKTTGSLVSSTFSSSL
KPTATFSCARAFSQTSSIMSKVFFD
↑SPP ↑MPP ↑MIP

B



C



6.11 Determination of the Sites of Cleavage of Pre-$F_1\beta$ by SPP

As described earlier, upon incubation of pre-$F_1\beta$ with SPP, several bands are seen after analysis by SDS-PAGE and fluorography, showing that SPP is cleaving this precursor protein at several different sites. However, one major band is seen and so the site of cleavage to form this major band was investigated. Pre-$F_1\beta$ was synthesised in the presence of [^{3}H]phenylalanine and processed *in vitro* by SPP. After Edman degradation of the reaction products, cycles were counted for ^{3}H radioactivity and the results shown in figure 40.

There is only one phenylalanine residue in the expected vicinity of the cleavage site, immediately N-terminal to the MPP cleavage site, so a single peak of radioactivity was expected from the degradation. Figure 40 shows the release of two peaks of radioactivity, at cycles 5 and 12. This must reflect the multiple cleavage sites within the presequence. The larger peak at cycle 12 is probably due to the major cleavage product, with processing occurring between two alanine residues 12 residues N-terminal to the MPP cleavage site (site SPP1 in figure 40). The smaller peak at cycle 13 may be due to one of the minor cleavage products which can be seen from SDS-PAGE and fluorography. In this case processing occurs between a proline and an alanine residue, 5 residues N-terminal to the MPP cleavage site (SPP2 in figure 40).

Figure 40 *Identification of SPP cleavage sites within the* Neurospora crassa *pre-F1β presequence*

Panel A

Sequence of the first 43 residues of pre-$F_1\beta$. Arrows show sites of cleavage by SPP and MPP. Residues radiolabelled for the Edman degradation analysis are underlined.

Panel B

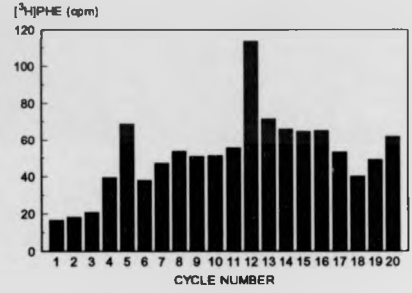
[^{3}H]phenylalanine-labelled pre-$F_1\beta$ was generated by *in vitro* transcription and translation and cleaved by incubation with SPP. The cleavage products formed were subjected to Edman degradation and counts determined for each cycle of the degradation.

A

MFKSGISAFARTARPSFAAASR
RAVRPAALNLRAPALSRFASS

SPP1 SPP2 MPP

B



6.12 Comparison of SPP Cleavage Sites

SPP cleavage sites within a variety of precursor proteins, both chloroplast and mitochondrial, have been determined by radiosequencing of the cleaved products. The cleavage of mitochondrial precursor proteins by SPP is unexpected, as many MPP cleavage sites have been determined and a number of common features deduced from a comparison of these sites. Mitochondrial protein presequences have a potential amphiphilic α-helical region followed by a more loosely structured region and the transition between these two regions has been proposed to be a recognition signal for MPP. MPP cleavage sites in general have an arginine residue at positions -2 or -3 which may play an important role in defining the exact position of the cleavage site (von Heijne *et al.*, 1989). A loosely conserved consensus sequence for SPP cleavage sites, (val/ile)-X-(ala/cys)↓ala, (based on the cleavage sites of stromal protein precursors) was proposed by Gavel and von Heijne (1990). The region around the cleavage site was also shown to have a high tendency for forming an amphiphilic β-sheet. However, more recently it has been suggested that chloroplast transit peptides are in general devoid of long stretches of secondary structure and may be flexible peptides which can interact with a number of different chaperones during the import pathway (von Heijne and Nishikawa, 1991). The consensus sequence does not occur in the sites determined here, suggesting that there are other important factors which control the site of cleavage. The most conserved feature of all of the SPP cleavage sites described here, both chloroplast and mitochondrial, is an alanine residue after the cleavage site. This occurs in every site except for cyclophilin, which has a serine residue at this position, and also in the suggested consensus sequence. Replacement of this residue in wheat pre-33K by a lysine residue resulted in a dramatic reduction in efficiency of cleavage by SPP, which also suggests that this residue is important in the cleavage reaction.

MPP and SPP cleavage sites appear to be very different in their structural requirements. Why, therefore, should SPP cleave mitochondrial protein precursors but not other proteins tested? Mitochondrial protein presequences, in addition to

chloroplast protein presequences, are thought to interact with several different chaperones during import into the mitochondrial matrix (Manning-Krieg *et al.*, 1991; Endo, 1991). Presequences could therefore be unfolded peptides which are accessible to processing peptidases. Many proteins may contain cryptic SPP recognition signals which are hidden within the protein. Mitochondrial presequences may have a more unfolded conformation than the majority of proteins, thus rendering them susceptible to cleavage by SPP. The inability of MPP to cleave any of the chloroplast proteins tested may suggest that MPP has more stringent requirements for certain structural features or that chloroplast transit peptides do not contain the required features for MPP recognition.

CHAPTER 7 - PROPERTIES OF AN SPP ACTIVITY FROM *CHLAMYDOMONAS REINHARDTII*

7.1 Introduction

Chlamydomonas reinhardtii is a unicellular green alga containing a single chloroplast (figure 41). *C. reinhardtii* chloroplast protein presequences have been compared with those of mitochondrial and higher plant chloroplast proteins (Franzen *et al.*, 1990) and seem to be more similar in some respects to mitochondrial presequences than those of higher plant chloroplasts in that they contain a potential amphiphilic α-helix, thought to be a critical feature in the targeting of proteins to mitochondria. However, they have also been suggested to contain an amphiphilic β-strand close to the SPP cleavage site, a possible characteristic of higher plant chloroplast precursor proteins. This leads to the question of whether the cleavage specificities of the SPP activities from pea and *C. reinhardtii* are similar, or whether maturation of precursors in these species occurs by differing mechanisms. This question was addressed by A. Creighton (MSc thesis) who showed that SPP activities from pea and *C. reinhardtii* were able to process chloroplast precursor proteins from both species in many cases. The study of the *C. reinhardtii* enzyme has therefore been extended by further purification of the enzyme and a study of the ability of the more highly purified enzyme to cleave various precursor proteins.

7.2 Chlamydomonas reinhardtii *SPP Activity*

A procedure for the partial purification of SPP activity from *Chlamydomonas reinhardtii* was developed by A. Creighton (MSc thesis) which gave a considerable purification when compared with the starting material. As the activity appeared to co-elute after both of the column steps used in the protocol with fewer proteins than was the case for SPP activity from pea, further development of a purification strategy was thought possible. The purification strategy developed is shown in figure 42.

Figure 41 *Schematic diagram of a cell of* Chlamydomonas reinhardtii

Light microscopy has shown that the cell is polar in structure with two anterior flagellae and a single cup-shaped chloroplast. Pyrenoids are found within the chloroplast, often surrounded by starch bodies. The cell wall is closely appressed to the plasma membrane and an eye spot and vacuoles can also be seen. (Adapted from "The *Chlamydomonas* Sourcebook", by E. H. Harris, 1989).

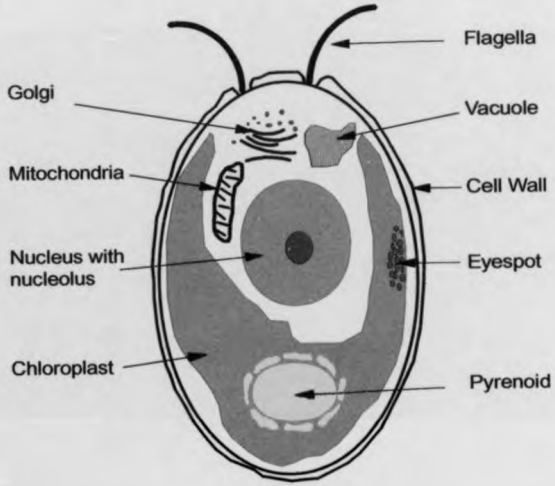
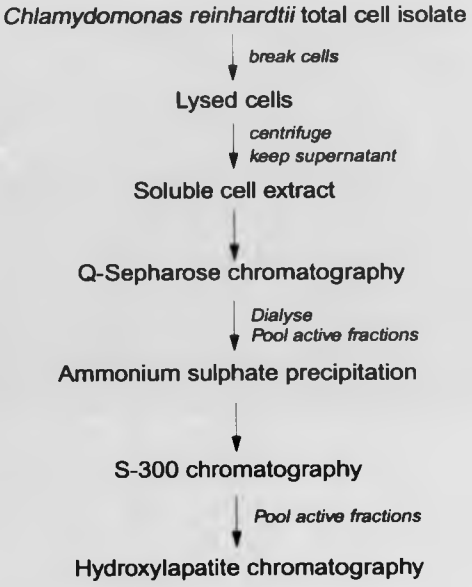

Flagella
Golgi
Vacuole
Mitochondria
Cell Wall
Nucleus with nucleolus
Eyespot
Chloroplast
Pyrenoid

Figure 42 *Strategy for the purification of SPP activity from* Chlamydomonas reinhardtii

Activity was purified and assayed as described in Materials and Methods.


Chlamydomonas reinhardtii total cell isolate
break cells
Lysed cells
centrifuge
keep supernatant
Soluble cell extract
Q-Sepharose chromatography
Dialyse
Pool active fractions
Ammonium sulphate precipitation
S-300 chromatography
Pool active fractions
Hydroxylapatite chromatography

7.2.1 *The Processing Activity Previously Identified in* C. reinhardtii *is Located in the Stroma*

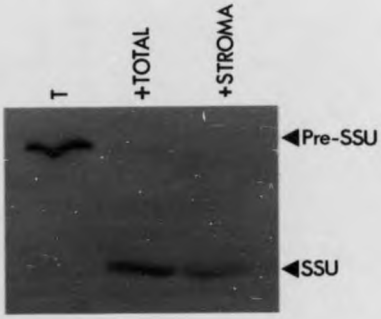
Due to the difficulties in obtaining intact chloroplasts from *C. reinhardtii*, previous work on the purification of SPP activity used a total soluble cell extract as a starting material. Before continuing this work, it was considered important to confirm that the activity observed is located in the chloroplast stroma as expected. A cell wall-deficient mutant (CC-400 *cw-15 mt*$^+$) of *C. reinhardtii* was obtained from the Duke University *Chlamydomonas* Culture Collection which allows the preparation of intact chloroplasts (Mason *et al.*, 1991; section 2.24.2), which in the wild type strain is complicated by the presence of the cell wall. A stromal extract was prepared by lysis of the intact chloroplasts (section 2.24.3) and compared with a total cell extract prepared as described in section 2.24.4. *In vitro*-synthesised radiolabelled *C. reinhardtii* pre-SSU was incubated with 20 µl of each of the extracts for 90 minutes at 27^{o}C to allow the processing reaction to occur (section 2.26). The reaction products were analysed by SDS-PAGE and fluorography as shown in figure 43. Both the total cell extract and the stromal extract contained SPP activity which processed pre-SSU to a lower molecular weight, corresponding to that of the mature sized protein. Activity purified from a total cell extract was shown to cleave pre-SSU at the correct site (A. Creighton, MSc thesis) and this activity is therefore located in the stroma.

7.2.2 *Partial Purification of SPP Activity From* C. reinhardtii

Previous work on the purification of *C. reinhardtii* SPP activity (A. Creighton, MSc thesis) involved the use of Q-Sepharose anion exchange chromatography followed by Sephacryl S-300 gel filtration chromatography to fractionate a total soluble cell extract from wild type *C. reinhardtii* strain 11/32b *mt*$^-$ (obtained from the Culture Collection of Algae and Protozoa, U.K.) which gave a significant purification and a good yield of activity and these steps were therefore included as the first steps of a protocol for further purification (section 2.24). Active

Figure 43 *Processing of pre-SSU from* Chlamydomonas reinhardtii *by a total soluble cell extract and a stromal fraction from* C. reinhardtii

Pre-SSU (lane T) was synthesised in a wheatgerm lysate system as described in section 2.19 and incubated with 20 µl of either a preparation of a total soluble cell extract (lane + total) or a stromal extract (lane + stroma) from *Chlamydomonas reinhardtii* (section 2.24) for 90 minutes at 27°C. The reaction products were analysed by SDS-PAGE and fluorography.


T
+TOTAL
+STROMA
Pre-SSU
SSU

fractions from the Sephacryl S-300 step were identified by their ability to process pre-SSU to the mature size, and pooled.

Pooled fractions from the Sephacryl S-300 column were loaded onto a hydroxylapatite column (1 x 10 cm; section 2.24.7) and eluted using a linear 30 ml gradient of 10 mM - 100 mM sodium phosphate buffer pH 8.0. Measurement of the optical density of each fraction at 280 nm revealed that very little protein was eluted from the column using this gradient and most of the protein loaded onto the column could only be removed using very harsh, denaturing conditions such as washing with NaOH. The fractions eluted using the sodium phosphate gradient were dialysed against 20 mM Tris-Cl pH 8.0 to ensure that assay conditions were consistent and SPP activity was assayed by the cleavage of pre-SSU from *C. reinhardtii* to the mature size (figure 44). SPP activity is therefore eluted from the column by the phosphate gradient, whereas most of the protein in the extract remains bound to the column. Very little protein was seen even on silver-staining of an SDS-PAGE gel upon which had been run 20 µl of each fraction, and so 200 µl of each fraction was concentrated by TCA precipitation (section 2.32), resuspended in 20 µl of sample buffer and again visualised by silver-staining of an SDS-PAGE gel (figure 44).

7.2.3 Analysis of the Purification Procedure

The extension of the previous protocol for the partial purification of *C. reinhardtii* SPP activity described here has resulted in the preparation of a highly purified fraction with no protein visible on a Coomassie-stained gel of the preparation. It has also emphasised the extremely low abundance of this enzyme: the most highly purified fractions required a concentration step before protein could been seen even by silver-staining, thus making purification of the enzyme to homogeneity a difficult proposition. After the three chromatography steps described, it was still not possible to assign the SPP activity to a protein band on a silver-stained gel and further purification will probably require an affinity chromatography step, using an immobilised precursor protein for example. The amount of enzyme present at each

Figure 44 *Hydroxylapatite chromatography of a* Chlamydomonas reinhardtii *protein extract*

SPP activity from *C. reinhardtii* was partially purified by Q-Sepharose and Sephacryl S-300 chromatography and the active fractions pooled. This pooled sample was chromatographed on a hydroxylapatite column as described in section 2.24 and fractions collected.

Panel A

200 µl of each fraction was subjected to TCA precipitation and the precipitated protein resuspended in 20 µl of protein sample buffer. Samples were analysed by SDS-PAGE followed by silver staining. Lane M - molecular weight markers, with sizes indicated. Lane Tot - total protein loaded on the column (20 µl). Fraction numbers are shown across the top.

Panel B

20 µl of each fraction (lanes 38-54) or the total extract loaded onto the column (lane Tot) was incubated with *in vitro* synthesised pre-SSU (lane T) from *Chlamydomonas renhardtii* for 90 minutes at 27°C. Samples were analysed by SDS-PAGE followed by fluorography. Fraction numbers are indicated across the top.

M
Tot
38
40
42
44
46
48
50
52
54
kDa
94
67
43
30
20
14
A

T
Tot
38
40
42
44
46
48
50
52
54
Pre-SSU
SSU

stage of the purification was determined by quantification of the amount of pre-SSU processed to the mature size by laser densitometry of the exposed film (table 1), but the nature of the assay means that the quantity is very approximate. In the later stages of purification, the amount of protein was too low to determine by the Bio-Rad protein assay method and so a specific activity could not be calculated. Overall, however, it is clear that the hydroxylapatite step may be very useful when a highly purified preparation of *C. reinhardtii* SPP is required.

7.3 Comparison of SPP activities from P. sativum *and* C. reinhardtii

Work by A. Creighton (MSc thesis) showed that SPP activities from pea and *C. reinhardtii* were able to cleave several chloroplast precursor proteins from both pea and *C. reinhardtii* to the mature (stromal proteins) or intermediate (thylakoid lumen proteins) size, with the exception of pre-SSU from *C. reinhardtii*, which was cleaved by pea SPP to an intermediate size only. This could be due to the unusually acidic sequence of amino acids around the SPP cleavage site in this precursor of Glu-Ala-Asn-Asp ↓ Met (von Heijne *et al.*, 1989).

The activity of the partially-purified SPP preparation against various thylakoid lumen protein precursors from pea and *C. reinhardtii* was tested. Active fractions obtained by hydroxylapatite chromatography were pooled and this preparation incubated with radiolabelled pre-23K and pre-33K from wheat and pre-33K from *C. reinhardtii* synthesised in a rabbit reticulocyte lysate *in vitro* translation system as described in section 2.26. These precursor proteins were also incubated with a pea SPP preparation for comparison purposes. Samples were analysed by SDS-PAGE and fluorography (section 2.20).

The results obtained (figure 45) show that in each case the precursor proteins are cleaved by both pea and *C. reinhardtii* SPP activities to produce the intermediate sized forms. This agrees with data obtained by A. Creighton using SPP prepared by Q-Sepharose chromatography and confirms that the activity cleaving pre-SSU from *C. reinhardtii* co-elutes with activities cleaving higher plant precursor proteins,

Table 1 *Partial purification of SPP activity from* Chlamydomonas reinhardtii

SPP activity from *C. reinhardtii* was purified and assayed as described in Materials and Methods. One unit of activity is taken as the amount of enzyme required to convert 20% of the precursor of SSU to the mature size, as determined by laser densitometry of autoradiographs from processing assays.

	Protein mg	Total activity U	Specific activity U/mg	Purification -fold	Yield %
Crude extract	100	12500	125	0	100
Q-Sepharose	9.6	10400	1083	8.7	83
Sephacryl S-300	1.2	8500	7083	56.6	68
Hydroxylapatite	<0.1	6700	>67000	>536	54

Figure 45 *Cleavage of thylakoid lumenal precursor proteins by SPP preparations from pea and* Chlamydomonas reinhardtii

Panel A

Radiolabelled wheat pre-23K was synthesised by *in vitro* transcription and translation in a rabbit reticulocyte lysate system (lane T) and incubated with 20 mM Tris-Cl pH 8.0 (lanes B), a Q-Sepharose eluate containing SPP activity from *C. reinhardtii* (lane Q), a hydroxylapatite eluate containing SPP activity from *C. reinhardtii* (lane H) or pea SPP activity (lane P) for 90 minutes at 27°C. Reaction products were separated by SDS-PAGE and visualised by fluorography.

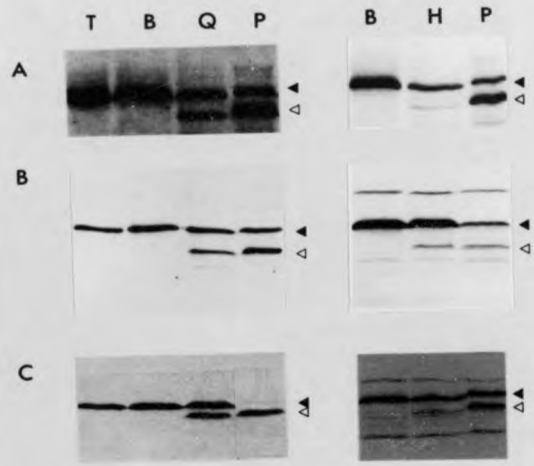
Panel B

As for panel A but using wheat pre-33K as the substrate.

Panel C

As for panel A but using *C. reinhardtii* pre-33K as the substrate.

In each case, filled arrows indicate the sizes of the precursor proteins and open arrows the sizes of the intermediate forms. Panels showing processing by the Q-Sepharose eluate were kindly donated by A. Creighton (Warwick).


T
B
Q
P
B
H
P
A
B
C

providing further evidence that SPPs from pea and *C. reinhardtii* have similar reaction specificities.

CHAPTER 8 - FINAL DISCUSSION

A highly purified SPP extract was prepared from pea chloroplasts by the modification of existing protocols, which enabled further characterisation of the properties and specificity of the enzyme. This was achieved by ammonium sulphate precipitation, gel filtration and anion exchange chromatography. The final enzyme preparation was purified around 1000-fold from the stromal extract used as the starting material and was highly active in its ability to remove the ETD from thylakoid lumen precursor proteins.

The site at which SPP cleaves stromal protein precursors has been determined previously for many proteins by the isolation of the protein from plant tissue and N-terminal sequencing of the protein. This is not possible in the case of thylakoid lumenal proteins as N-terminal sequence data would reveal only the TPP cleavage site and no information can be obtained on the SPP cleavage site from the cDNA sequence. The sites of SPP cleavage within three thylakoid lumen presequences were therefore determined by synthesising radiolabelled precursor proteins *in vitro*, incubating with a partially-purified SPP preparation to produce the intermediate forms and performing an Edman degradation analysis, with the radioactive counts being determined for each cycle of the degradation. This approach allowed the identification of the SPP cleavage sites within wheat pre-23K and pre-33K and *Silene pratensis* pre-PC. Surprisingly, considering the wide variety of residues found at the SPP cleavage sites of stromal precursor proteins (von Heijne *et al.*, 1989), SPP was shown to cleave all three of the thylakoid lumenal proteins between a positively charged residue and an alanine residue. It is not yet clear whether the same enzyme cleaves both stromal protein and thylakoid lumen protein precursors, but there is as yet no evidence for the existence of more than one enzyme.

The identification of these SPP cleavage sites enabled a mutagenesis study of the sites to be carried out. The residues on either side of the SPP cleavage site within the wheat pre-33K presequence were altered using an oligodeoxynucleotide-directed mutagenesis approach. The four mutant precursor proteins created (arg → met, arg → glu, arg → ala and ala → lys) were all cleaved by SPP in an *in vitro* processing assay but with widely varying efficiencies and rates. Time course analyses of the cleavage of the mutant precursor proteins by varying concentrations of SPP showed that the arg → met and arg → ala mutants were both cleaved at approximately 15-20% of the rate of cleavage of non-mutated pre-33K, suggesting that the size of the residue before the cleavage site is unimportant but that the loss of the positive charge in this position causes a decrease in the rate of processing. The arg → glu mutant was cleaved very slowly in the processing experiments, which is probably due to the presence of the negative charge. The region just N-terminal to the SPP cleavage site in most precursor proteins tends to be enriched in positively charged residues (von Heijne *et al.*, 1989) and the mutagenesis results suggest that these residues play a role in determining the efficiency of cleavage by SPP. The SPP cleavage site of the ala → lys mutant was almost completely blocked, as very little cleavage of this precursor could be seen even using the most concentrated SPP sample. This shows that this residue is also very important in determining the efficiency of cleavage, but it is not possible to draw any further conclusions about the role of this residue. Lysine is a larger residue than alanine and is also positively charged and either of these properties could be important in the susceptibility of the precursor to SPP cleavage. The introduction of the lysine residue in this position also means that the presequence contains two positively charged residues adjacent to each other, which could cause a distortion in the structure of the presequence and thus alter the conformation around the cleavage site. Clark and Lamppa (1991) showed that, in the case of pre-LHCP, three residues around the SPP cleavage site specified the efficiency of cleavage by SPP, and the time course analyses of the mutant precursors described here suggest that the same situation applies to the processing of pre-33K. In order to investigate

the requirements for particular amino acids in these positions in greater detail, a wider variety of mutant precursor proteins would need to be synthesised, with different residues substituted in the positions mutated here, and in other positions close to the cleavage site. The effect of these mutations on the secondary and tertiary structure of the presequence could be investigated using computer modelling techniques, although these cannot as yet predict the structures of individual presequences accurately, making any conclusions drawn from this unreliable. The secondary structure of several presequences of mitochondrial and *E. coli* precursor proteins has been shown to vary depending on the environment of the presequence (Roise *et al.*, 1986; Endo and Schatz, 1988; Briggs *et al.*, 1986), further complicating this kind of analysis.

Each of the mutated precursor proteins is cleaved by SPP to a size which appears to be the same as that of the non-mutated intermediate 33K, as shown by SDS-PAGE. However, this gel system cannot resolve proteins whose sizes differ by a very small amount, and so it was possible that the mutated precursors were cleaved at a different site to the genuine SPP cleavage site. The cleavage sites of the three arginine mutants were therefore determined using a radiosequencing approach, similar to that originally used to determine the site of SPP cleavage of non-mutated pre-33K. In each case, SPP was seen to cleave very specifically at the authentic cleavage site, and the alteration of this arginine residue does not therefore have any effect on the site of cleavage of pre-33K. This must be specified by other unidentified features within the presequence. The cleavage site of the ala → lys mutant precursor protein could not be determined by this approach due to the extremely low efficiency of cleavage of this precursor, which did not allow the production of enough of the intermediate sized protein for the sequencing reaction.

The effect of the mutations on the import of pre-33K into intact isolated pea chloroplasts was investigated. Pre-33K can be imported *in vitro* into chloroplasts in a presequence-dependent manner and is located in the thylakoid lumen after import (James *et al.*, 1989). Each of the mutated precursor proteins was found to be imported into chloroplasts, localised to the thylakoid lumen and processed to the mature size in

a manner indistinguishable from that of non-mutated pre-33K. The substitution of a single residue in these positions therefore has no effect on the import properties of the precursor proteins. No information can be obtained in this way about the cleavage of the precursors by SPP in intact chloroplasts, however, as it is not known whether pre-33K must be cleaved by SPP before being translocated across the thylakoid membrane or whether the full precursor can cross the membrane.

The presequences of imported chloroplast and mitochondrial proteins have been compared (von Heijne *et al.*, 1989) and, despite superficial similarities in protein import into chloroplasts and mitochondria, have been found to have distinctly different properties. This is not unexpected, as in plant cells many proteins must be targeted specifically to either the chloroplast or the mitochondrion. The processing of these precursor proteins may be expected to be similarly specific for either SPP or MPP; however, recent work has shown that a protease in a crude stromal extract (Whelan *et al.*, 1991) or in a partially-purified pea SPP preparation (A. Creighton, MSc Thesis) is able to process mitochondrial precursor proteins, apparently to the mature size. An extension of these investigations to include other mitochondrial precursor proteins has revealed that this cleavage event, at least in some cases, yields products of different sizes to those produced by MPP cleavage. Radiosequencing of the cleavage products of two precursors indicated that in each case cleavage occurred N-terminal to the authentic MPP cleavage site, *ie* within the mitochondrial presequence. The identity of the protease responsible for this cleavage reaction was investigated initially by column chromatography of a stromal extract, which demonstrated that the protease cleaving the mitochondrial precursor proteins co-eluted with the SPP activity of the extract after both gel filtration and ion exchange chromatography. This strongly suggests that the SPP activity itself is responsible for the cleavage reaction. The unknown protease was shown to be inhibited by divalent metal ion chelators only, again consistent with the protease being SPP (Robinson and Ellis, 1984).

The ability of SPP to cleave mitochondrial presequences shows that these presequences must contain the features required for recognition by SPP as well as for cleavage by MPP. A comparison of mitochondrial and chloroplast presequences may therefore give some information about the necessary requirements for SPP cleavage of a protein, in particular of the secondary structure as there appears to be very little primary structure similarity between presequences. However, as dicussed earlier, the prediction of structure is still very inaccurate and preliminary attempts gave different results depending on the method of prediction, making the interpretation of these results difficult. SPP appears to cleave mitochondrial presequences just after a structure identified by von Heijne *et al.* (1989), which was suggested to correspond to an α-helix with a ridge of hydrophobic amino acids spiralling clockwise along the helix. No such structure is predicted for chloroplast presequences, and so this structure may not be significant in recognition by SPP. The only feature which occurs at most of the cleavage sites determined is an alanine residue immediately C-terminal to the site of SPP cleavage. This is found at five of the six cleavage sites determined, suggesting that it plays some role in the processing reaction. This residue is not generally found at the cleavage sites of stromal precursor proteins (von Heijne *et al.*, 1989), which may indicate that there is more than one species of SPP, with different species having slightly different reaction specificities. However, there is no direct evidence for this and so this point remains to be clarified.

The cleavage of mitochondrial precursor proteins should also be assayed using purified SPP to enable comparisons between the rates of cleavage by SPP and by MPP. This would give an indication of the efficiency of cleavage by SPP compared with MPP, which is not possible with a partially-purified preparation, as the amount of enzyme present cannot be accurately measured. The partially-purified SPP preparation does, however, cleave some mitochondrial precursors more efficiently than many chloroplast precursors, indicating that the cleavage of mitochondrial precursor proteins is highly efficient.

An SPP activity can also be detected in the unicellular green alga *Chlamydomonas reinhardtii* which is capable of processing chloroplast precursor proteins from both *Chlamydomonas reinhardtii* and pea (A. Creighton, MSc Thesis). This enzyme has been shown to be located in the stroma and an improved protocol has been developed for the partial purification of this enzyme from a total soluble cell extract of *C. reinhardtii*. The band on a silver-stained gel corresponding to the enzyme can not be identified, probably due to its extremely low abundance, but a highly purified preparation, essentially free of other detectable protease activities, has been made. This extract is capable of cleaving precursor proteins from *C. reinhardtii* and pea, demonstrating the similarities of specificity between the SPP activities of the two species, as expected from previous work using a less purified *C. reinhardtii* SPP preparation (A. Creighton, MSc thesis).

In summary, the recognition of various precursor proteins, both chloroplast and mitochondrial, for cleavage by SPP seems to depend on structural features both at the cleavage site and in other areas of the presequence. Mutations at the cleavage site can dramatically reduce the extent of cleavage by SPP, but do not appear to affect the site of cleavage, which therefore must be specified by features more distant from the cleavage site. These features must also be present in mitochondrial presequences, and a consideration of both mitochondrial and chloroplast presequences may thus be useful in identifying possible recognition features.

Some of the work presented in this thesis has been included in the content of the following publications:

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CHAPTER 9 - REFERENCES

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